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1959 Iodine Research Award Nominations Requested Prior to January 1, 1959

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1959 Chilean Iodine Educational Bureau, Inc., Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine.

The award consists of $1,000 and a diploma setting forth the reasons for selection of the recipient. It may be presented each year at the annual meeting of the ASSOCIATION.

Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1959 Award, *nominations must be received on or before January 1, 1959.*

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION's Council. The present committee includes Justin L. Powers, chairman; F. F. Blicke, E. A. Brecht, John E. Christian, Stanley G. Mittelstaedt, Lloyd M. Parks, and Linwood F. Tice.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. Travel expense to the meeting at which the award is to be made is provided for up to $250.

The award is now in its eleventh year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist; the second recipient was Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University, and the third was Dr. C. P. Leblond, Professor of Anatomy, McGill University, Montreal, Canada. The fourth award was shared jointly by Dr. George Moore of the University of Minnesota Medical School and Dr. Moses Ashkenazy, Chief of Neurosurgery, Veterans Hospital, Houston, Tex. Dr. Vernon H. Wallingford, Mallinckrodt Chemical Works, St. Louis, Missouri, was the recipient of the fifth award. Dr. Jack Gross, Associate Professor of Anatomy, State University of New York, College of Medicine in Brooklyn, received the sixth award and Dr. Dominick Papa, Schering Corporation, Bloomfield, N. J., received the seventh award. The eighth award went to Dr. John E. Christian, Professor of Pharmaceutical Chemistry, School of Pharmacy, Purdue University, Lafayette, Indiana. The ninth award was conferred on Dr. James O. Hoppe, Research Pharmacologist, Sterling-Winthrop Research Institute, Rensselaer, New York, during the 1957 Convention of the AMERICAN PHARMACEUTICAL ASSOCIATION. No award was made in 1958.

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NOTICE

PAPERS FOR SECTION MEETINGS OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

The next annual meeting of the American Pharmaceutical Association will be held in Cincinnati, Ohio, April 19 to 24, 1959. Titles and abstracts (not to exceed 200 words) of papers must be submitted to the appropriate Section secretary not later than February 1, 1959, or preferably earlier, in order to assure a place on any Section program.

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Purification of Fructose on Ion Exchange Resins*

By IRA B. CUSHING and RAYMOND V. DAVIS

A method is described for the purification of fructose made by fermentation of mannitol with *Acetobacter suboxydans*. Some observations on the behavior of fructose on strongly basic ion exchange resins are recorded.

IT HAS BEEN KNOWN since 1904 that *Acetobacter suboxydans* converts mannitol to fructose in high yield, but until recently only small scale laboratory fermentations had been reported. At Abbott Laboratories submerged fermentation studies have provided large amounts of fructose-containing beer (1). This paper reports methods for the treatment of such beer with ion exchange resins to obtain crystalline fructose for pharmaceutical use.

Two main problems were encountered. One was the complete removal of color formers, and the other was suppression of side reactions such as the adsorption and epimerization of sugar on the ion exchange resin. Two methods were evolved for getting syrups of acceptable purity, of which the second proved to be more practical.

The untreated beer was a pale yellow solution containing about 18 per cent fructose. The yellow pigments were readily removable by carbon, but there remained colorless organic impurities which caused formation of more color when the solution was evaporated in vacuum. Crystals were obtained from such an evaporated solution by crystallization from alcohol, but in very poor yield, and a 10 per cent aqueous solution of such crystals turned deep red if heated for fifteen minutes at 114° for sterilization. To be acceptable, the crystals had to be pure enough that the solution would develop no color when heated under these conditions.

Since the beer contained 0.05 per cent KH_2PO_4 as the only significant inorganic constituent, the solution seemed ideally suited to treatment with ion-exchange resins. The nitrogen content of only 0.5 mg. per liter indicated a low content of amino acids, which are often the commonest color formers. We soon learned that most color formers were adsorbed on strongly basic anion exchange resins such as Amberlite IRA-400[1] or Dowex 1,[2] but were poorly adsorbed on the more weakly basic resins. A simple sequence of alternating sulfonic acid cation exchange resins and quaternary ammonium exchangers, no matter how many times repeated, failed to produce suitably pure crystals.

A solution (2) to the problem was found when we observed that the fructose solution after passage through two columns, Amberlite IR-120[3] followed by IRA-400, could be autoclaved for thirty minutes at 114° to produce bright yellow condensation products between fructose and the unknown impurities. These condensation products were completely adsorbed on a freshly prepared bed of IRA-400 in the hydroxyl form. A

* Received May 15, 1958, from Abbott Laboratories, Research Division, North Chicago, Ill.

Numerous analyses for reducing sugar were provided by the Control Department, and fermentation beers were supplied by the Microbiology Department and the Development Laboratories.

[1] Product of Rohm and Haas, Philadelphia, Pa., abbreviated IRA-400.

[2] Product of Dow Chemical Co., Midland, Mich.

[3] Product of Rohm and Haas, Philadelphia, Pa., abbreviated IR-120. Amberlite IR-112 mentioned in the patent is no longer made.

final passage over a regenerated and washed column of IR-120 reduced the pH of the purified solution. This was called Process I. (See Table I.)

It was soon apparent that Process I was too costly. Process II was then devised which eliminated the autoclave step. It was observed that the condensation of color formers with fructose was accomplished readily at room temperature at a pH between 9 and 11. The pH chosen was high enough to cause condensation of impurities to take place rapidly before bacterial growth could occur, and yet low enough that alkaline degradation of sugar was negligible. It will be shown in the experimental section that the alkaline catalysis was best obtained by addition of some alkali followed by passage over a small amount of IRA-400. During the alkaline reaction there was rapid development of intense light absorption at low wavelengths. The cations were then removed on IR-120 and the color formers on IRA-400. (See Table I.)

TABLE I.—SUMMARY OF PROCESS I AND PROCESS II

	Step	Purpose
Process I	IR-120	Remove cations
	IRA-400	Remove anions and color formers
	Autoclave	Condense persistent color formers with fructose
	IRA-400	Remove condensation products
	IR-120	Reduce pH
Process II	pH elevation and/or IRA-400	Condense color formers with fructose
	IR-120	Remove cations
	IRA-400	Remove anions and condensation products

EXPERIMENTAL

Measurement of Absorbance.—Absorbance was measured in the Beckman DU spectrophotometer in a 1-cm. cell at the arbitrarily chosen wavelength 350 mμ. The absorbance of fructose solution falls from very high values in the ultraviolet, without any peaks in the curve, to very low values at the red end of the visible spectrum. (The weak maximum at 279 mμ can be seen only in solutions of pure crystals.) At the wavelength chosen, solutions colorless to the eye gave measurable readings in a 1-cm. cell, yet the yellow color of the autoclaved solution in Process I could be measured without dilution. Ten per cent (w/v) solutions of fructose crystals which appeared colorless to the eye when viewed in 500-ml. or 1000-ml. bottles had an absorbance of less than 0.04 at 350 mμ. On the American Public Health Association scale (3) originally set up for the examination of drinking water, a fructose solution with an absorbance of 0.04 gave a reading of about 10.

Losses on Quaternary Ammonium Base Resins.—By far the largest part of the loss of fructose occurred by adsorption of each passage of the fructose solution over the strongly basic resin. Because some impurities were so poorly adsorbed we found it necessary to use the rather large ratio of 150 ml. basic resin to treat 120 Gm. fructose. Approximately 9 Gm. fructose is held by this much resin in the hydroxyl form. Therefore, the average loss per column passage was approximately 7 to 8% of the total fructose, but sometimes varied considerably from this figure for unknown reasons.

It has been shown (4, 5) that the adsorption and epimerization of fructose is negligible on the carbonate form of the strongly basic resin. However, the carbonate form was much less efficient in removing color formers. In Table II is shown the comparison of the hydroxyl and carbonate forms of the resin.

TABLE II.—EFFECT OF FORM OF BASIC RESIN ON QUALITY OF FRUCTOSE SYRUP

Resin Form	Forerun 1st IRA-400, ml.	Yield of 4 Columns + Autoclave, %	Final Autoclave Color of Syrup 350 mμ	$[\alpha]^{25}_{D}$ RS, deg.
OH	120	86	0.06	−84.5
$^1/_2$ CO_2	96	92	0.31	−86.6
CO_2	75	90	1.0	−88.3

The half carbonated resin was obtained by mixing equal volumes of the carbonate and hydroxyl forms and pouring the mixture into a column. The volume of the forerun from the first IRA-400 column, before the first sugar effluent appeared, decreased as fructose adsorption decreased until in the case of the fully carbonated form the volume of forerun was equal to the holdup volume of the resin bed. Column 4 of Table II shows the absorbance of a sample of finished syrup, without a crystallization step, adjusted to 10% w/v fructose concentration and heated at 114° for fifteen minutes.

[α] RS as a Measure of Epimerization and Fragmentation.—In column 5 of Table II is given [α]RS, a specific rotation based on reducing sugars, which was found very useful in following the effects of basic resin columns on fructose. It is calculated by dividing the observed rotation by the per cent reducing sugar obtained by the alkaline copper reagent. The substances in solution inert to oxidation by alkaline copper do not enter the calculation and the possible optical rotations of any impurities is ignored. If fructose were the only oxidizable substance present [α]RS at 25° and $c = 10$ would be −90°. All rotations were observed with the D line of sodium. The correct values of specific rotation of fructose at any given concentration and temperature were obtained from the work of Tsuzuki, Yamazaki, and Kagami (6).

Because there are oxidizable impurities in varying amounts in untreated beers, values of [α]RS are only comparable when taken on the same beer.

It was found by paper chromatography that glucose accounted for only one-third to one-fifth of the oxidizable impurities in finished resin-treated beer. Some oxidizable impurities are present in the carbon treated and filtered beer, but more are formed by fragmentation of fructose on IRA-400. The acids known (4, 5) to be formed from fructose by IRA-400 should be held by the resin under our conditions.

Effect of Flow Rate on Epimerization.—Other workers (7–9) have shown that conversion of fructose to glucose and mannose is very extensive at the long contact times with strongly basic resins. We were able to minimize the loss due to such conversion by operating at rapid flow rates. To obtain such rates we used resin beds only 40 to 50 cm. in depth which is less than usually considered necessary for efficient ion removal.

In Table III an example of the effect of different flow rates taken from Process I is illustrated.

TABLE III.—EFFECT OF FLOW RATE ON YIELD AND ROTATION

Flow Rate, ml./Min.	Yield to Syrup, %	$[\alpha]_D^{25}$ RS, deg.
20	74	−75.3
50	86	−85.2

In this example the column of basic resin contained 300 ml. of resin which had a hold-up volume of roughly 150 ml. At a flow rate of 50 ml. fructose solution per minute the average contact time of a fructose molecule with the resin was three minutes, compared to seven and one half minutes at the lower rate.

Although the general inverse relation of degree of epimerization and fragmentation with flow rate was repeatedly observed, many anomalies were uncovered which showed that there are unknown variables in this reaction. For example, in Table IV fructose

TABLE IV.—EFFECT OF FLOW RATE OVER IRA-400 ON ROTATION

Flow Rate, ml./Min.	Observed Rotation, deg.	$[\alpha]_D^{25}$ RS, deg.
25	−13.5	−84.0
12	−13.2	−83.5
50	−13.5	−84.8

beer treated with a cation exchanger was passed over a bed of IRA-400 in the hydroxyl form. After each rate was established, 400 ml. of effluent was discarded and then a 100-ml. portion was collected for assay. The variations in [α]RS were in the expected direction but the difference between the values at the lowest flow rate (contact time twelve minutes) and the highest rate (contact time three minutes) was much less than expected.

Another observation was that in pilot plant runs contact times of fifteen minutes produced no greater drop in [α]RS than seven minutes did in laboratory runs.

Within the range of pH of our IRA-400 effluents, 8.5 to 9.5, we found no relation of pH to losses. However, in the first IRA-400 column of Process II, where the pH of the input solution was 10 or over, adsorption was sharply reduced.

Regeneration of Anion Exchange Resin.—In the first process, the autoclaved and cooled fructose syrup was passed over the resin, and the resin was then regenerated with 0.5 *N* sodium hydroxide. After approximately ten such cycles the resin began to show reduced capacity for adsorption of the colored condensation products, and after still more cycles the ion exchange capacity was also reduced and disintegration of the resin beads to finely divided amorphous material was plainly visible. This breakdown was completely prevented by exhausting the resin with 1 *N* sulfuric acid after each use, before regenerating with alkali. This practice was continued in the second process even though it was observed that the amount of colored degradation products, removable by sulfuric acid but not removable by alkali, was markedly reduced. It is not known in this latter case if exhaustion by sulfuric acid after each passage of fructose was necessary for maximum resin life. It is therefore possible that exhaustion with acid after, say, every fifth cycle might suffice.

Condensation of impurities by Catalysis at Alkaline pH.—The elevation of pH necessary for condensation of impurities in Process II was obtained in three ways: (*a*) by passing the beer through a short column of IRA-400 resin; (*b*) by adding sodium hydroxide to pH 11 and allowing the solution to stand at room temperature; and (*c*) by adding sufficient sodium hydroxide to elevate the pH only to about 10, filtering after a short waiting period, and passing this alkaline solution through a short column of IRA-400. Method (*a*) was impractical because a flocculent precipitate formed when the pH rose and this partially plugged the column and reduced the flow rate. The third method consistently resulted in the best purification. The short column of resin seemed to function catalytically to complete the condensation of potential color formers rapidly under the mildest conditions.

During the alkaline reaction there was rapid development of intense absorption at short wavelengths, accompanied by a fall in optical rotation. Table V shows the changes in these two variables,

TABLE V.—CHANGE IN ABSORBANCE AND ROTATION IN 0.04 *N* NaOH

Time, Min.	Absorbance, 350 mμ		—Rotation, deg.—	
	Beer	Crystals	Beer	Crystals
0	0.03	0.005	−14.0	−14.0
10	1.9	0.04	−13.3	−13.8
60	3.6	0.06	−13.1	−13.8
180	3.8	0.10	−13.0	−13.8

compared with the corresponding changes in a solution of fructose crystals, adjusted to the same initial rotation. Absorbances of beer were read by diluting a portion 10 times and multiplying the dilution reading by 10. The small change in rotation of the solution of fructose crystals was due to the change in volume caused by addition of 5 *N* NaOH as well as to a small shift in rotation with pH.

Examination of Impurities in Finished Syrup.—In order to reveal some of the other constituents by paper chromatography, the syrup was applied as one microliter spots containing, according to the rotation, about 200 mcg. fructose. After the chromatograms had been developed by downward flow with a suitable mixed solvent, the location of spots was determined by the method of Trevelyan, Procter, and Harrison (10). Greater uniformity of development was obtained by dipping the paper in silver nitrate in acetone (as in the original method) drying, then dipping the paper in the solution of sodium hy-

droxide in alcohol instead of spraying this reagent as in the original method. One normal ammonia was found adequate to remove unused silver nitrate from the paper, and did not weaken the paper as did the 6 *N* ammonia of the original method. Surprisingly, we found no other developer which would reveal as many minor constituents as this reagent.

By these techniques, it was shown that glucose in a finished syrup from Process I, having an $[\alpha]_D^{25}$ RS of −86° was only 0.5 to 1.0%. In Process II the glucose dropped to 0.25–0.5%. It was also found that residual mannitol was present only to the extent of 0.5% of the weight of the fructose.

For a more detailed examination of the inert constituents, portions from each step of process I containing about 20 Gm. of fructose were obtained. The fermentable sugars were removed by yeast fermentation and the remaining unfermented material was passed through 120 ml. IRA-400 to remove traces of fructose and glucose. The nonfermentable substances showed a gradual rise, with no one step consistently responsible for the increase. Chromatograms of nonfermentables from starting beer and finished syrup developed twenty-four hours by downward flow with acetone, butanol, water (7:2:1) showed 5 spots having R_f, the speed of movement relative to fructose, of 0.15, 0.79, 0.91, 1.47, 1.71. There were, in addition, in finished syrup many minor spots forming almost a streak throughout the developed area. The spot at 0.15 was present in beer but increased during processing. The spot at 0.91 gave a test for aldose or ketose with aniline oxalate as developer. When 1.23 Gm. of nonfermentables were passed a second time over 120 ml. freshly regenerated and washed IRA-400 (OH^-), the effluent no longer gave a spot at this position. The spot at R_f of 0.91, therefore, seems to be a nonfermentable sugar which is very poorly adsorbed on IRA-400. Its position corresponds to the speed of movement of psicose which has been reported (8) to be formed by action of IRA-400 on glucose, but no authentic sample was available for comparison. The R_f for the same finished syrup developed with acetic acid, butanol, and water (5:2:1) were 0.14, 0.95 (mannitol), 1.17, 1.47, 1.90. In Process II the spots due to inerts were in the same locations, but the total amount in finished syrup dropped to 2 to 3% of the total solids.

Detailed Experimental Procedure.—A procedure for Process I has not been included because it was the less practical of the two procedures.

To 800 ml. of fermentation beer containing about 18% fructose was added 1% w/v of an activated carbon such as Darco G-60,[4] and 1% w/v Supercel-Hyflo.[5] The mixture was stirred for thirty minutes and was filtered. To the filtered beer was added 3 ml. 5 *N* NaOH and the solution was stirred fifteen minutes, 1% Supercel-Hyflo was added and stirring was continued another fifteen minutes. The solution was filtered and the filtrate was passed through a bed of IRA-400 16 cm. deep and 50 ml. in volume at a flow rate of 30 ml. per minute. The beer was followed with sufficient distilled water to wash the remaining fructose solution from the column. The beer was then passed through a well-washed bed of IR-120, H^+ form, depth 48 cm. and volume 150 ml.,[6] at a flow rate of 30 ml./min. and was followed with sufficient distilled water rinse. A second IRA-400 (OH^-) column, depth 48 cm. and volume 150 ml. was prepared. If the column had previously been used for fructose purification it was exhausted first with 1 *N* sulfuric acid, and, after most of the excess acid had been washed out of the column, was regenerated with sodium hydroxide and was then washed free of excess alkali. The fructose solution was passed through the column at a flow rate of 30 ml./min. and was followed with the usual distilled water rinse. The fructose solution was evaporated under vacuum at an inside pressure of 10 mm. mercury, with a jacket or bath temperature of not over 45°, until the syrup was about 85% total solids and 15% water. One volume denatured ethanol was added to the syrup and the mixture was stirred and warmed gently until a uniform solution occurred. Stirring was continued and when the temperature fell to 40° a few fructose crystals were added. Stirring was continued at room temperature until crystallization was complete, which was generally in less than ten hours. The crystals were removed by vacuum filtration and were washed with some fresh ethanol, and were dried under vacuum without heat. Table VI gives a summary of the yield at each step calculated from the observed rotation and assuming that fructose is the only material present giving a significant contribution to the observed rotation.

TABLE VI.—SUMMARY OF RESIN TREATMENT OF FRUCTOSE BY PROCESS II

	Vol.	pH	α^{27}	Fructose, %	Fructose, Gm.
After 3 ml. 5 *N* NaOH	750	10.0	-14.3^{35}	15.9	118
IRA-400, 50 ml.	860	10.2	-12.3^{35}	13.7	117
IR-120, 150 ml.	1020	3.0	-10.5^{35}	11.7	119
IRA-400, 150 ml.	1040	9.3	-9.5^{35}	10.6	110

Syrup weight 134 Gm. approx. %F = 110/134 = 82%. Wt. crystals 85 Gm. (77%). Overall yield 72%. $[\alpha]_D^{25}$ = −89° (*c* = 10). Autoclave color, 350 mμ, 10% solution = 0.025.

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[4] Product of Atlas Powder Co., Wilmington, Del.

[5] Product of Johns Manville Corporation.

[6] This volume is now known to be larger than necessary.

Correlation Between Anticonvulsant Activity and Plasma Concentration of Ethanol*

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Ethanol was found to exhibit anticonvulsant activity in nontoxic doses as measured by four of six well-standardized methods. The effective dose of ethanol, as determined by the various tests, was found to be linearly related to its plasma concentration. The initial increase in low-frequency electroshock seizure threshold, induced by a single large dose of ethanol, was followed by a significant decrease. The central nervous system tends rapidly to accommodate to the ethanol milieu so that the electroshock seizure threshold does not exactly parallel the plasma ethanol concentration. The significance of these observations is discussed.

IT IS WELL KNOWN that the acute administration of ethanol depresses the central nervous system. Nevertheless, ethanol is also known to precipitate seizures in epileptic patients (1–3) and, consequently, this agent is generally withheld from persons afflicted with this disease (4). Despite this apparent discrepancy, comparatively little attention has been directed to the anticonvulsant properties of ethanol or to the effects of a single dose of this agent on the brain excitability of laboratory animals. Except for a few cursory reports on the ability of ethanol to elevate the threshold for electroshock convulsions (5–8) and to modify the pattern of maximal electroshock seizures (8, 9), there is a lack of precise experimental data on the anticonvulsant properties of this agent. Therefore, it was thought important to test ethanol by a battery of well-standardized methods. In order to correlate the anticonvulsant activity of ethanol with its level in the extracellular fluid, the concentration of plasma ethanol also was determined. The results obtained provide the basis for this report.

METHODS

Adult male albino mice obtained from the Carworth Farms (CF #1 strain) were used as experimental animals. They were maintained on Purina Laboratory Chow and allowed free access to food and water except during the short time they were removed from their cages for testing. The ethanol was administered orally as a 20% (w/v) solution and the animals were tested at the times indicated below.

Anticonvulsant Assay Methods.—Anticonvulsant potencies (ED_{50}) were determined by a battery of six assay procedures, four electrical and two chemical. The tests based on electrically-induced convulsions measure the ability of a drug to modify the pattern of maximal electroshock seizures evoked by supermaximal current (MES test; 50 ma. alternating current, 0.2-second stimulus duration), to elevate the threshold for minimal electroshock seizures (alternating current; 0.2-second stimulus duration) in normal mice (a. c. EST test) and in hyponatremic (low-threshold) mice (HET test), and to elevate the threshold for low-frequency electroshock seizures (l. f. EST test) induced in mice by unidirectional current delivered at an intensity twice threshold (0.2-millisecond duration, 3-second stimulus duration, six pulses per second). The tests based on chemically-induced convulsions measure the ability of a drug to modify the pattern of maximal seizures evoked by pentylenetetrazol (Metrazol) injected intravenously (MMS test; 38 mg./Kg.) and to elevate the threshold for minimal seizures induced by the subcutaneous injection of Metrazol (Met. test; 85 mg./Kg.). In addition, neurotoxicity (TD_{50}) was determined on the basis of signs of minimal overt neurological deficit. Except that a Grass Stimulator (model S4B) was used for the l. f. EST test, the details of the various procedures and the end points employed for determination of acute neurotoxicity in mice have been described elsewhere (10–13) and need not be repeated here. For the determination of ED_{50} or TD_{50}, groups of 10 to 12 mice were given various doses of ethanol orally and tested at the time of peak effect (thirty minutes), as previously determined by the MES test, until at least three points were established between complete protection or toxicity and no protection or toxicity. The results obtained were then plotted on logarithmic probability paper and a regression line was fitted to the plotted points by eye. From this plot of the data, the ED_{50} or TD_{50} was determined and the protective indexes ($PI = TD_{50}/ED_{50}$) were computed; 95% fiducial limits were calculated by the method of Litchfield and Wilcoxon (14).

In a separate series of experiments, 100 mice were randomly divided into two equal groups. One group was given orally 4,500 mg./Kg. of ethanol as a 20% (w/v) solution, and the other group was given the requisite volume of water; the low-frequency electroshock seizure threshold was determined for both groups at the times indicated in Fig. 2. The mice were shocked at various voltages by the staircase procedure (15), and the voltage required to

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evoke convulsions in 50% of each group was determined; the 95% fiducial limits were calculated by the method of Litchfield and Wilcoxon (14). The results are presented as the threshold ratio (threshold of drug group/threshold control group). The above experiment was repeated with a fresh group of 100 mice and a 6,000 mg./Kg. dose of ethanol.

Determination of Plasma Ethanol.—Each ED_{50}, the TD_{50}, and the 4,500 mg./Kg. dose of ethanol was given to groups of four to eight animals; at the time of peak drug effect or at the times indicated in Fig. 2, the mice were sacrificed and their pooled plasma analyzed for ethanol by the residual titration method of Levine and Bodansky (16).

RESULTS

The data obtained are summarized in Table I from which it can be seen that the TD_{50} of ethanol was 2,952 mg./Kg.; and that the drug was effective by all anticonvulsant tests employed. Except for the l. f. EST test, ethanol was approximately 2.5 times more effective against chemically induced seizures than it was against a similar type of electrically induced seizure. For example, ED_{50}s by the MMS and Met. tests were 700 and 1,886 mg./Kg., respectively, whereas the ED_{50}s by the MES and a. c. EST tests were 1,886 and 4,500 mg./Kg., respectively. Ethanol was approximately 3 times as effective against seizures induced by low-frequency stimulation with interrupted unidirectional current (l. f. EST test) than it was against seizures induced by conventional electroshock procedures (a. c. EST test).

The efficacy of ethanol by the two Metrazol tests can also be seen from the protective indexes (PI's) listed in Table I. The PI's by the MMS and Met. tests were 4.22 and 1.57, respectively, whereas the PI's for the MES and a. c. EST tests were 1.57 and 0.66, respectively. The effectiveness of ethanol against seizures induced by low-frequency electroshock is shown by the fact that the PI by the l. f. EST test is well above unity (2.08), whereas the PI's by the a. c. EST and HET tests are well below unity (0.66 and 0.63, respectively); this indicates that ethanol is effective by these two latter tests only in neurotoxic doses.

As shown in Table I, the concentration of plasma ethanol which resulted from the administration of the TD_{50} and each ED_{50} varied from 27.1 mg./100 ml. of plasma after a nontoxic dose of 700 mg./Kg. to 330 mg./100 ml. after a toxic dose of 4,700 mg./Kg. The relation between the dose of ethanol and the concentration of plasma ethanol is shown in Fig. 1. Statistical analysis of the data indicates that the relation between the dose of ethanol and the concentration of plasma ethanol is linear.

In order to determine the effect of ethanol on the sensitivity of the central nervous system, a dose of 4,500 mg./Kg. of ethanol was given orally to mice, and the low-frequency EST and plasma ethanol concentration were determined at various times during the next forty-eight hours. The results obtained are illustrated in Fig. 2 from which it may be seen that maximal anticonvulsant effect occurs thirty minutes after ethanol administration, at which time the threshold of treated mice is 3.57 times that of control animals. The threshold gradually declined, and by the 12th hour after ethanol administration it was somewhat lower than normal. Since this suggested that an optimal single dose of ethanol might be followed by hyperexcitability, the effect of 1,500, 3,000, and 6,000 mg./Kg. of ethanol on l. f. EST also was studied. Although hyperexcitability again was observed to follow the initial depression, it was significant only following the larger dose (see open circles Fig. 2). Thus, the threshold ratio and 95% fiducial limits eight, twelve, and fifteen hours after the 6,000 mg./Kg. dose of ethanol were 0.90 (0.79–1.04), 0.80(0.73–0.88), and 0.87(0.74–1.03), respectively. Plasma ethanol reached a peak concentration (323 mg./100 ml. plasma) one hour after

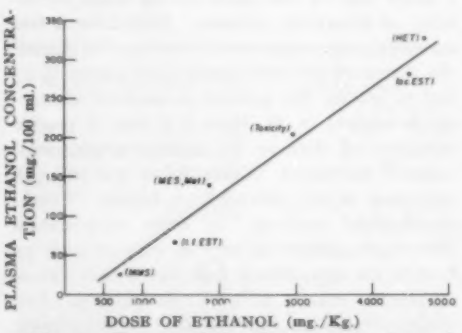

Fig. 1.—The correlation between the dose of ethanol and the concentration of plasma ethanol. The dose of ethanol in mg./Kg. is shown on the abscissa and the plasma ethanol concentration (mg./100 ml.) on the ordinate. The regression line was fitted to the plotted points by the method of least squares.

TABLE I.—CORRELATION BETWEEN ANTICONVULSANT ACTIVITY AND CONCENTRATION OF PLASMA ETHANOL

		Tests					
		Electrical				Chemical	
	Toxicity	MES	a. c. EST	HET	l. f. EST	MMS	Met.
Dose 50[a] mg./Kg.	2952	1886	4500	4700[b]	1420	700	1886
	2708–3218[c]	1684–2112[c]	3947–5130[c]	4234–5217[c]	1214–1661[c]	574–854[c]	1572–2263[c]
Protective index (PI)		1.57	0.66	0.63	2.08	4.22	1.57
		1.41–1.74[c]	0.55–0.79[c]	0.58–0.70[c]	1.73–2.50[c]	3.38–5.28[c]	1.31–1.88[c]
Plasma ethanol mg./100 ml.	205	140	284	330	66.6	27.1	140

[a] Given orally as a 20% (w/v) solution of ethanol.
[b] Based on animal weight one and one-half hours after the intraperitoneal administration of the isosmolar glucose.
[c] 95% fiducial limits.

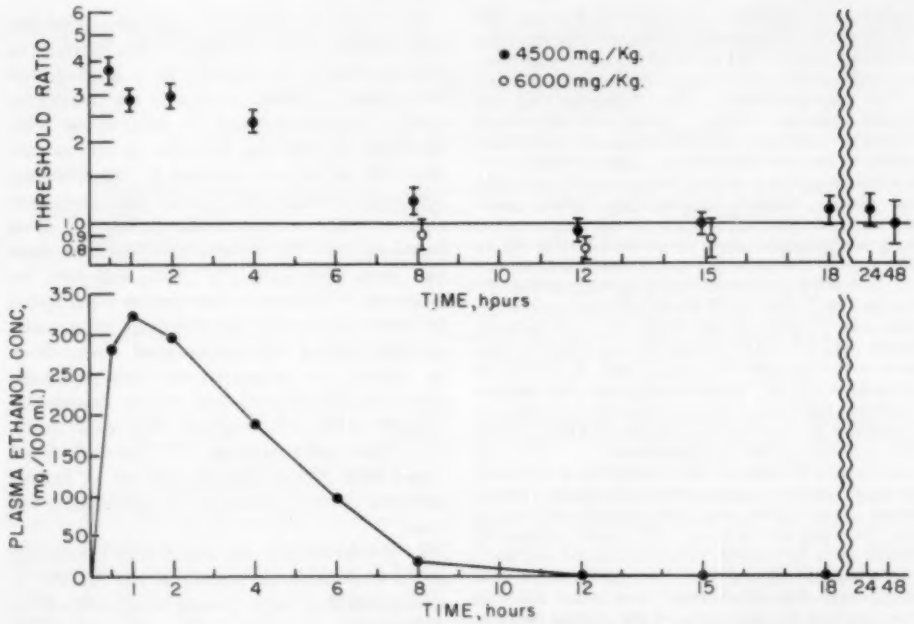

Fig. 2.—The threshold for low-frequency electroshock seizures and the plasma ethanol concentration at various times after the oral administration of 4,500 and 6,000 mg./Kg. of ethanol. Time in hours is indicated on both the upper and lower abscissa. Seizure threshold ratio (threshold of ethanol group/threshold of control group) is shown on the upper ordinate (logarithmic scale). The filled circles indicate the threshold ratio at the indicated times after the oral administration of 4,500 mg./Kg. of ethanol; the open circles, the ratio eight, twelve, and fifteen hours after 6,000 mg./Kg. of ethanol. Vertical bracketed lines indicate 95% fiducial limits. The plasma ethanol concentration (mg./100 ml.) after the 4,500 mg./Kg. dose is shown on the lower ordinate.

drug administration and was no longer detectable by the chemical procedure employed at twelve hours and beyond.

DISCUSSION

The results presented indicate ethanol is effective by all anticonvulsant tests employed and can modify seizure pattern as well as elevate seizure threshold. It is 2.7 times more effective against maximal Metrazol seizures than against maximal electroshock seizures. Likewise, ethanol is 2.4 times more effective in elevating the threshold for Metrazol than that for electroshock seizures. The efficacy of ethanol against Metrazol seizures, in altering pattern and elevating threshold, is of particular interest. In previous publications (13, 17), mention has been made of the unique ability of trimethadione, as compared to other antiepileptics, to modify maximal chemoshock seizure pattern as compared to maximal electroshock seizure pattern. By these two tests trimethadione has the lowest ratio (MMS ED_{50}/MES ED_{50}) of any antiepileptic examined; in contrast, diphenylhydantoin, which effectively modifies the pattern of seizures (variously induced) but which is incapable of elevating threshold for Metrazol seizures, has the highest ratio. Indeed, all clinically useful hydantoins have ratios well above unity, whereas the other antiepileptics have ratios well below unity. This separation of antiepileptics into two classes probably reflects the fact that hydantoin derivatives more effectively prevent seizure spread than elevate seizure threshold. The ratio for ethanol is well below unity (0.37) and approaches that for trimethadione (0.31); ethanol would thus appear to act similar to those drugs which both prevent seizure spread and elevate threshold.

The fact that neurotoxic doses of ethanol were required to elevate the threshold in mice by conventional electroshock procedures is in agreement with the relatively low margin of safety observed by Tainter and co-workers (5) in rabbits and Driver (6) in rats. Thus, the PI's by the a. c. EST and HET tests are 0.66 and 0.63, respectively. On the other hand, the efficacy of ethanol by the MES, l. f. EST, MMS, and Met. tests is shown by PI's of 1.57, 2.08, 4.22, and 1.57, respectively. These favorable indexes reemphasize the fact that current methods of laboratory assay as yet have only limited value for the prediction of clinical antiepileptic potency or of drug specificity for particular types of seizures.

There is a direct relation between the log dose of ethanol and the plasma concentration of ethanol (Fig. 1). Furthermore, ethanol appears to penetrate other body fluids rapidly. For example, the plasma ethanol concentration in hyponatremic (low-threshold) animals (HET test) was found to be 330 mg./100 ml. of plasma, whereas the ethanol concen-

tration in the intraperitoneal fluid was 349 mg./100 ml. of fluid. This somewhat higher concentration of ethanol in the peritoneal fluid than in the plasma, in all probability, is not statistically significant.

The data presented in Fig. 2 suggest that the central nervous system rapidly accommodates ("adapts"), at least to some extent, to its ethanol milieu so that the threshold for seizures induced by low-frequency electroshock does not exactly parallel the plasma ethanol concentration. Thus, peak elevation of l. f. EST occurs thirty minutes after drug administration (see upper half of Fig. 2) at which time the plasma ethanol concentration has yet to reach a peak level (see lower half of Fig. 2). On the other hand, peak plasma ethanol concentration occurs one hour after drug administration at which time l. f. EST already has started to decline. Soon after the last of a large dose of ethanol is eliminated by the experimental animal, the seizure threshold falls below normal. As shown in Fig. 2, significant hyperexcitability is present twelve hours after a 6,000 mg./Kg. dose of ethanol.

According to Lennox (18), susceptible individuals do not exhibit convulsions during the period of maximum concentration of plasma ethanol, but during the "sobering up" process. The results presented herein are in agreement with this concept and indicate a biphasic effect follows the administration of a single large dose of ethanol. The initial effect is characterized by depression of the central nervous system as shown by the marked increase in the threshold for seizures induced by low-frequency electroshock stimulation; this initial depression is then followed by a short period of excitation as shown by the 20% decrease in threshold. McQuarrie and Fingl (8) have shown that seizure threshold, forty-eight hours after fourteen days of chronic ethanol treatment, is reduced by 50%. More recently, these workers (19) have reported a significant decrease in Metrazol seizure threshold occurs eight hours after a single dose of ethanol. If similar alterations in seizure threshold occur in man following the ingestion of alcohol, one would expect a marked reduction in seizure threshold to occur during the "sobering up" period. Hence, susceptible persons would be more likely to exhibit convulsions after imbibing alcohol. This interpretation provides a valid physiological reason for restricting the use of alcoholic beverages in epileptic patients.

SUMMARY

The anticonvulsant potencies (ED_{50}s) of ethanol were determined in mice by a battery of six assay procedures. The dose which produced evidence of minimal neurological deficit in 50% of animals (TD_{50}) was determined and protective indexes (PI = TD_{50}/ED_{50}) were calculated. In addition, the concentrations of plasma ethanol obtained after administration of the TD_{50}, each ED_{50}, and a 4,500 mg./Kg. dose of ethanol were also measured. The results are as follows:

1. The TD_{50} of ethanol, determined in mice thirty minutes after oral administration, is 2,952 mg./Kg.

2. Ethanol is effective by all anticonvulsant tests employed and modifies seizure pattern as well as elevates threshold. It is most potent when tested for ability to modify the pattern for maximal seizures induced by intravenous Metrazol and by electroshock, and to elevate the threshold for seizures induced by subcutaneous Metrazol and by low-frequency electrical stimulation with unidirectional pulses of short duration; the PI's derived from data by these four tests were 4.22, 1.57, 1.57, and 2.08, respectively. Ethanol is least potent when tested for ability to elevate the threshold for minimal seizures induced by conventional electroshock in normal or hyponatremic (low-threshold) mice; the PI's derived from values obtained by these two tests were 0.66 and 0.63, respectively.

3. The concentrations of plasma ethanol varied from 27.1 to 330 mg./100 ml. of plasma and were linearly related to the logarithm of the dose.

4. Evidence was presented which indicates the central nervous system tends rapidly to accommodate to high plasma concentrations of ethanol.

5. Significant hyperexcitability was observed twelve hours after the oral administration of 6,000 mg./Kg. of ethanol. The possible clinical implications of this observation were discussed.

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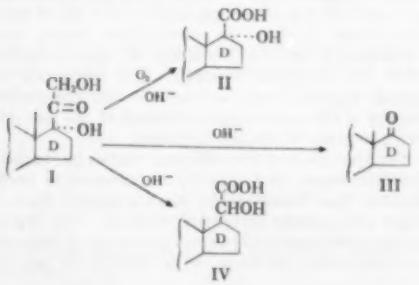
The Kinetics of the Base-Catalyzed Degradation of Prednisolone*

By DAVID E. GUTTMAN† and PETER D. MEISTER

The rate of disappearance of the dihydroxyacetone function of prednisolone was studied in homogenous alkaline solution. The degradation of prednisolone exhibited a first-order dependency on the steroid concentration and occurred with the concomitant appearance of acidic and neutral steroidal products. The exclusion of air from the system was found to decrease the rate of degradation. This slowing of the rate was apparently due to the elimination of an oxygen-dependent reaction which normally formed an acidic steroidal product. The results suggested that the destruction of prednisolone in aqueous alkali resulted from at least three parallel pseudo first-order reactions. The hydroxide-ion dependencies of the reactions were investigated and the hydroxide-ion catalytic constants were estimated.

THE REACTIVITY of the dihydroxyacetone side chain of adrenocorticosteroids has been rather well documented. Base-catalyzed rearrangements and eliminations have been shown to occur under both aerobic and anaerobic conditions.

In the presence of air, the predominant reaction appears to involve an oxidative cleavage of the side chain (I) to yield the corresponding etianic acid (II). Velluz, *et al.* (1), for example, reported that deoxycorticosterone acetate and 21-acetoxypregn-5-en-3β-ol-20-one were converted by ethanolic potassium hydroxide in the presence of air to the corresponding etianic acids. Herzig and Ehrenstein (2) showed similar results using 6β,21-diacetoxypregn-4-ene-3,20-dione as the substrate.



In the absence of air, two reactions predominate yielding the 17-ketosteroid (III) and the hydroxyacid (IV). Transformations of this nature were reported by Mason (3), studying the degradation of cortisone, and by Wendler and Graber (4) utilizing 3α,17α,21-trihydroxypregnane-11,20-dione.

Although these investigations show that therapeutically important steroids such as hydrocortisone and prednisolone are susceptible to degradative processes, the kinetics of such reactions have received little apparent attention. Chulski and Forist (5) investigating the effect of some solid buffering agents on prednisolone in aqueous solution demonstrated by a rate study that magnesium oxide caused a first-order degradation of the steroid. A paper chromatographic examination of their reaction mixture suggested that prednisolone was converted predominantly to 11β,17α-dihydroxy-3-ketoandrosta-1,4-diene-17β-carboxylic acid and to a lesser degree to 11β-hydroxyandrosta-1,4-diene-3,17-dione.

In the present investigation, the base-catalyzed degradation of prednisolone in aqueous solution was studied in some detail. As would be expected, the kinetics of the degradation was found to be relatively complex. No attempt was made to specifically separate the components of a reaction mixture. However, a broad separation of the neutral steroidal components from those exhibiting acidic characteristics was achieved by extracting the alkaline reaction mixture with chloroform. With this method of separation, and by utilizing ultraviolet spectroscopy and the triphenyltetrazolium chloride assay, it was possible to follow the rate of disappearance of the dihydroxyacetone function of prednisolone and the rate of appearance of nonreducing neutral and acidic steroidal products. Rate studies were conducted at a number of different hydroxide-ion concentrations, both in the presence and absence of air, and indicated that at least three parallel first-order reactions were involved in the degradation of prednisolone.

EXPERIMENTAL

The experimental method consisted essentially of: *1* allowing an alkaline solution of prednisolone to degrade at constant temperature and withdrawing aliquots at time intervals, *2* extracting the aliquot

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with chloroform, *3* determining the concentration of undegraded prednisolone by a tetrazolium assay on the chloroform extract, and *4* determining the amount of acid product formed by a spectrophotometric assay of the extracted aqueous phase.

Preparation of the Reaction Mixture.—Initially it was attempted to introduce prednisolone as a solid to the reaction medium. This proved unsatisfactory due to the slow rate of solution of this steroid. The use of a solution of the steroid in dimethylacetamide (DMA) overcame this difficulty and was subsequently used in all experiments. Usually 50 mg. of prednisolone in 1 ml. of DMA was added to a flask containing 200 ml. of NaOH solution. Prior to the addition of the steroid, the reaction medium was allowed to attain the temperature at which the study was made. The flask was agitated vigorously until a homogeneous solution resulted and was then placed in a constant-temperature bath. The experiments reported herein were conducted at 35°. In the case of the anaerobic studies, relatively air-free conditions were attained by using freshly boiled, deionized water which was thoroughly flushed with nitrogen immediately before use. An atmosphere of nitrogen was maintained over the reaction mixture at all times.

Extraction Procedure.—An aliquot of the reaction mixture, usually 10 ml., was withdrawn and immediately placed in a 125-ml. separatory funnel. Twenty ml. of cold (4°) chloroform was added and the funnel was shaken. After separation of the phases, the chloroform layer was allowed to drain through a pledget of glass wool into a 100-ml. volumetric flask. The aqueous phase was extracted with three additional 20-ml. portions of chloroform which were also collected in the volumetric flask. The solution was made to volume with chloroform. The aqueous solution remaining in the funnel was drained into a 100-ml. volumetric flask. The funnel was rinsed at least three times with deionized water and the rinsings collected in the volumetric flask. Deionized water was added to the fiducial mark of the flask. The extraction procedure provided a method of separating the components of the reaction medium into two classes; acidic material which was not extracted from the aqueous environment, and neutral material which was chloroform extractable.

Measurements.—The absorbances of the chloroformic and aqueous solutions were determined at a wavelength of 248 mμ. Light absorption at this wavelength is specific for the conjugated A-ring ketone group of delta steroids. The absorbance measurements thus served as a check on the stability of this chromophore. The concentration of acidic material, formed in the course of the degradation was determined from the absorbance of the diluted aqueous residue remaining after the chloroform extraction. In calculating the concentration of acidic material, an absorptivity of 42 was assumed. This was considered to be a legitimate assumption since it was determined that both prednisolone and the corresponding etianic acid, for example, had absorptivities of approximately 42 at this wavelength in aqueous solution.

The concentration of undegraded prednisolone was determined by evaporating an appropriate aliquot of the diluted chloroform extract to dryness and performing a tetrazolium assay on the residue. This assay is specific for the dihydroxyacetone side chain of the steroid. Here the residue was dissolved in 20 ml. of absolute alcohol. One milliliter of triphenyltetrazolium chloride reagent (1% triphenyltetrazolium chloride in absolute alcohol) was added followed by the addition of 1 ml. of tetramethylammonium hydroxide solution (1 ml. of a 10% aqueous solution diluted to 10 ml. with absolute alcohol). The color was allowed to develop in the dark for exactly thirty minutes. The development was then quenched by the addition of 1 ml. of glacial acetic acid and the absorbance of the resulting solution was determined at 485 mμ on a Beckman Model DU spectrophotometer. A standard solution of prednisolone in absolute alcohol was carried through the identical procedure and was used as a color reference in calculating the concentration of steroid in the unknown sample.

RESULTS AND DISCUSSION

Aerobic Studies.—Data typifying the results obtained in these studies are tabulated in Table I. These results were obtained when prednisolone was degraded in the presence of air at 35° in 0.01*N* NaOH. The stability of the A-ring chromophore was indicated by the sum of the absorbances of aqueous and chloroformic solutions remaining essentially constant over a relatively long period of time. Significant side-chain degradation did occur as evidenced from the results of the tetrazolium assays. It will be shown that this degradation was first-order with respect to the prednisolone concentration. This table also shows that concomitant with the disappearance of prednisolone was the formation of acidic material. The rate of formation of acid product was however less than the rate of disappearance of prednisolone. For example, the disappearance of 0.193 mg. of prednisolone from 1 ml. of solution which occurred in seventy-two hours was accompanied by the formation of approximately 0.160 mg. of acidic material. The formation of neutral material must account for the remainder. Results of this nature were obtained at the five different degrees of alkalinity studied.

The formation of two different classes of reaction products emphasized that the degradation of prednisolone was considerably more complex than a single step, pseudo first-order reaction. The degradation could conceivably involve a series of consecutive first-order reactions, or a number of parallel

TABLE I.—THE AEROBIC DEGRADATION OF PREDNISOLONE AT 35° IN 0.01 *N* NaOH

Time, Hr.	A_{CHCl_3}[a]	A_{H_2O}[b]	Prednisolone Concn., mg./ml.	Acid Concn., mg./ml.
0	0.980	0.000	0.193	0.00
1	0.900	0.070	0.173	0.016
2	0.820	0.134	0.154	0.031
3	0.779	0.196	0.140	0.046
5	0.682	0.296	0.111	0.069
6	0.580	0.337	0.101	0.079
7	0.540	0.370	0.091	0.087
72	0.270	0.671	0.000	0.160

[a] Absorbance of chloroform solution at 248 mμ.
[b] Absorbance of aqueous solution at 248 mμ.

first-order reactions, or a combination of consecutive and parallel reactions occurring to form more than one reaction product.

If the actual mechanism involved parallel first-order reactions such as,

$$P \xrightarrow{k_a} A$$
$$P \xrightarrow{k_n} N$$

where P, A, and N represent prednisolone, an acid product, and a neutral product, respectively, and k_a and k_n represent the corresponding rate constants then,

$$-\frac{dP}{dt} = k_a P + k_n P \qquad \text{(Eq. 1)}$$

$$= kP \qquad \text{(Eq. 2)}$$

where $k = k_a + k_n$ (Eq. 3)

and $\ln (P_o/P) = kt$ (Eq. 4)

or $P = P_o e^{-kt}$ (Eq. 5)

where P_o is the initial concentration of prednisolone and P is the concentration at any time t. The overall reaction is thus simple first order with respect to prednisolone. Also,

$$\frac{dA}{dt} = k_a P \qquad \text{(Eq. 6)}$$

$$= k_a P_o e^{-kt} \qquad \text{(Eq. 7)}$$

and by integration,

$$A = -\frac{k_a P_o e^{-kt}}{k} + \text{constant} \qquad \text{(Eq. 8)}$$

$$A = A_o + \frac{k_a}{k} P_o (1 - e^{-kt}) \qquad \text{(Eq. 9)}$$

where A_o is the initial concentration of the acid product and A is the concentration at any time t. If

$$A_o = 0 \qquad \text{(Eq. 10)}$$

then

$$A = \frac{k_a}{k} P_o (1 - e^{-kt}) \qquad \text{(Eq. 11)}$$

A similar expression can be derived for the neutral product.

Equation 11 expresses a linear relationship between the concentration of the acid reaction product and the function $(1 - e^{-kt})$. It will be recognized that when t is zero, the function has a value of zero. At infinite time the function has a value of one. To check the fit of the experimental data to this relationship it was first necessary to determine the value of k, the rate constant describing the overall degradation. Figure 1 illustrates the classical method of determining a first-order reaction. Here $\log_{10}$(prednisolone concentration) was plotted *versus* time. The curves obtained at five different concentrations of hydroxide ion exhibited the linearity characteristic of first-order reaction kinetics. The values of the slopes were calculated by the method of least squares, and from the slope values the rate constants were calculated. The

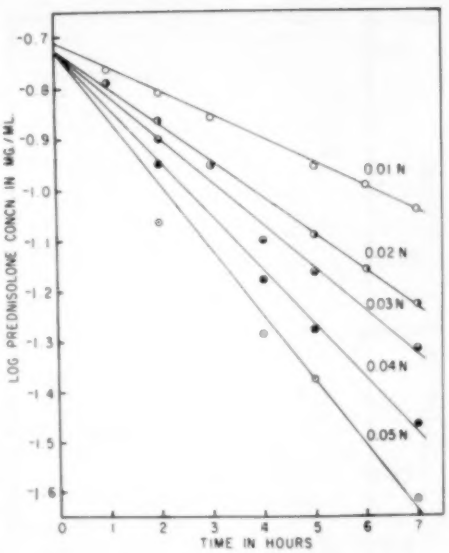

Fig. 1.—The disappearance of prednisolone from aqueous solution under aerobic conditions at various normalities of NaOH at 35°.

values of $(1 - e^{-kt})$ could then be calculated, the values of k and t now being known, and the relationship expressed in Eq. 11 could be checked. Figure 2 illustrates this plot. Here the concentration of acidic material formed when prednisolone was degraded at 35°, in 0.01 *N* NaOH in the presence of air was plotted *versus* $(1 - e^{-kt})$. A linear plot was obtained. The value of k_a, the rate constant describing the formation of acidic material, was calculated from the slope since by Eq. 11,

$$\text{slope} = \frac{k_a}{k} P_o \qquad \text{(Eq. 12)}$$

The value of k_n, the rate constant characterizing the formation of neutral material, was obtained by subtracting k_a from k, according to Eq. 3. This treatment was applied to the results obtained at the four other hydroxide-ion concentrations studied and the results are summarized in Table II where the appropriate rate constants are tabulated as a function of the hydroxide-ion concentration.

It should be emphasized that although the data fits the postulated expressions quite well, this does not necessarily imply that only two reactions are involved in the degradation. The mathematical

TABLE II.—RATE CONSTANTS FOR THE BASE-CATALYZED DEGRADATION OF PREDNISOLONE AT 35° IN THE PRESENCE OF AIR

Normality of NaOH	k, Hr.$^{-1}$	k_a, Hr.$^{-1}$	k_n, Hr.$^{-1}$
0.01	0.108	0.090	0.018
0.02	0.171	0.137	0.034
0.03	0.233	0.181	0.052
0.04	0.258	0.200	0.058
0.05	0.293	0.230	0.063

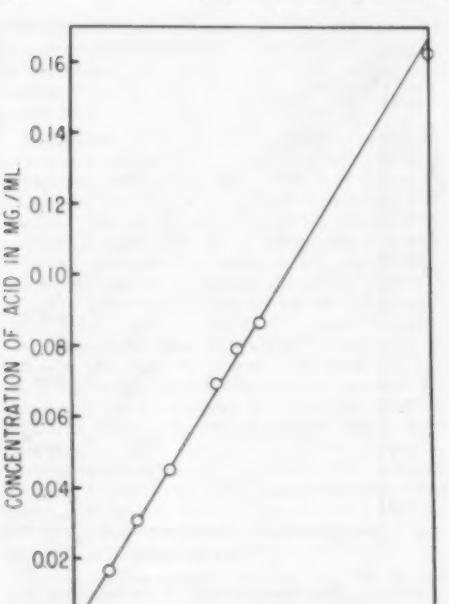

Fig. 2.—The appearance of acidic product during the aerobic degradation of prednisolone in 0.01 *N* NaOH at 35°.

treatment of the data will not differentiate between reactions which yield acidic products or between those reactions which form neutral products. The results do show, however, the formation of two classes of reaction products.

Anaerobic Studies.—Data typifying the base-catalyzed degradative behavior of prednisolone in the absence of air are tabulated in Table III.

Here it is seen that the anaerobic degradation of prednisolone is qualitatively similar to that observed in the presence of air, *viz.*, concomitant appearance of acidic and neutral products with the disappearance of prednisolone, as well as no detectable A-ring degradation. The rates of degradation under anaerobic conditions were studied at five concentrations of hydroxide ion and the results are summarized in Fig. 3. This plot shows that under anaerobic conditions the degradation was first-order with respect to prednisolone. Figure 4 demonstrates the fit of the data to the relationship expressed by Eq. 11. The rate constants were calculated in the manner described previously and are tabulated in Table IV.

A comparison of Tables II and IV reveals that when the reaction mixture was exposed to air, the disappearance of prednisolone occurred much more

Table III.—The Degradation of Prednisolone at 35° in 0.01 *N* NaOH in the Absence of Air

Time, Hr.	A_{CHCl_3}	A_{H_2O}	Prednisolone Concn., mg./ml.	Acid Concn., mg./ml.
0	0.930	0.00	0.206	0.00
1	0.878	0.046	0.187	0.011
2	0.858	0.069	0.178	0.016
3	0.840	0.088	0.168	0.021
5	0.820	0.123	0.153	0.029
6	0.795	0.142	0.146	0.034
7	0.772	0.160	0.138	0.038
24	0.610	0.323	0.070	0.077
48	0.523	0.424	...	0.101

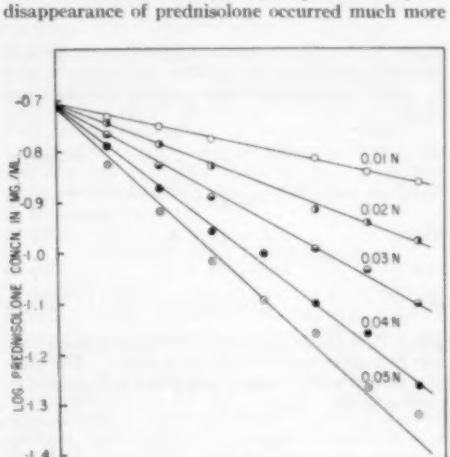

Fig. 3.—The disappearance of prednisolone from aqueous solution in the absence of air at various normalities of NaOH at 35°.

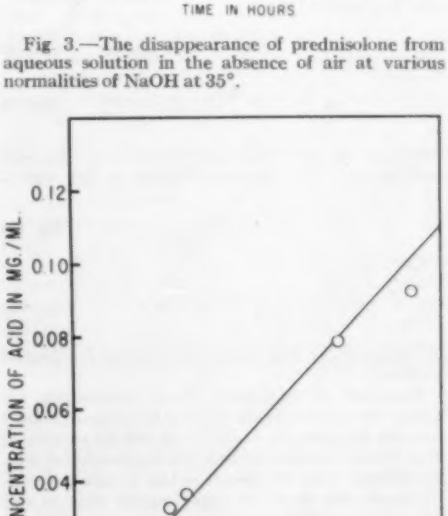

Fig. 4.—The appearance of acidic product during the anaerobic degradation of prednisolone in 0.01 *N* NaOH at 35°.

TABLE IV.—RATE CONSTANTS FOR THE BASE-CATALYZED DEGRADATION OF PREDNISOLONE AT 35° IN THE ABSENCE OF AIR

Normality of NaOH	k, Hr.$^{-1}$	k_a (Anaerobic), Hr.$^{-1}$	k_n, Hr.$^{-1}$
0.01	0.0541	0.0311	0.0230
0.02	0.0924	0.0531	0.0393
0.03	0.129	0.071	0.058
0.04	0.178	0.103	0.075
0.05	0.196	0.120	0.076

TABLE V.—THE RATE CONSTANTS FOR THE AEROBIC ACID-FORMING REACTION INVOLVED IN THE DEGRADATION OF PREDNISOLONE AT 35°

Normality of NaOH	k_a (Aerobic), Hr.$^{-1}$
0.01	0.0589
0.02	0.0839
0.03	0.110
0.04	0.097
0.05	0.110

rapidly. For example, at a hydroxide-ion concentration of 0.01 *N*, the rate constant for the overall degradation obtained under anaerobic conditions was approximately one-half the value of that obtained when no precautions were taken to exclude air from the system. Furthermore, the rate constant characterizing the formation of acidic material was markedly decreased by the exclusion of air while the rate constant representing the formation of neutral product appeared to be unaffected, considering the experimental error, by the presence or absence of air. A conclusion can thus be drawn that at least two reactions were involved in forming steroidal acid material from prednisolone under aerobic conditions. One reaction was dependent on the presence of oxygen, while the other occurred both in the presence and absence of air. Since k_a incorporates essentially two rate constants, the value of the constant representing the aerobic acid-forming reaction, k_a (aerobic), can be approximated by subtracting the value of k_a (anaerobic) from k_a. The values of k_a (aerobic) obtained at the various normalities of NaOH are tabulated in Table V.

In summary, the evidence obtained thus far suggests that the base-catalyzed degradation of prednisolone resulted from at least three parallel pseudo first-order reactions. Two or more oxygen-independent reactions formed neutral and acidic products while at least one oxygen-dependent reaction formed an additional acidic product.

The Hydroxide-Ion Dependencies of the Reactions.—The relationship between the observed rate constant and the hydroxide-ion concentration in a base-catalyzed reaction can usually be expressed by

$$k_{obs.} = k_{OH} - (OH^-)^n \qquad \text{(Eq. 13)}$$

where $k_{obs.}$ is the observed rate constant, k_{OH^-} is the hydroxide-ion catalytic constant, (OH^-) is the hydroxide-ion concentration, and n is an integer representing the order of the reaction with respect to hydroxide ion. The value of n can be determined graphically by a plot of log $k_{obs.}$ *versus* log (OH^-) since,

$$\log k_{obs.} = \log k_{OH^-} + n \log (OH^-) \qquad \text{(Eq. 14)}$$

Knowing n, the value of k_{OH^-} can also be determined graphically by plotting $k_{obs.}$ *versus* $(OH^-)^n$ according to Eq. 13.

In Fig. 5, the log of the observed rate constants k_a (aerobic), k_a (anaerobic), and k_n are plotted *versus* the log of the hydroxide-ion concentration. The values of k_a obtained under anaerobic conditions were used in this plot since they were thought to be more accurate than those obtained in the aerobic studies. The curves obtained for k_a (anaerobic) and k_n appear to be linear. The slopes were determined graphically to be 0.93 and 0.94, respectively, indicating that these reactions in all probability were first-order with respect to hydroxide ion. In the case of k_a (aerobic), the plot is not linear indicating the possibility of a changing order with respect to hydroxide ion. Here the slope appears to decrease from approximately 0.5 to 0.0 as the hydroxide-ion concentration increases over the range studied. A rate constant expression for this oxygen-dependent reaction which might account for such behavior is:

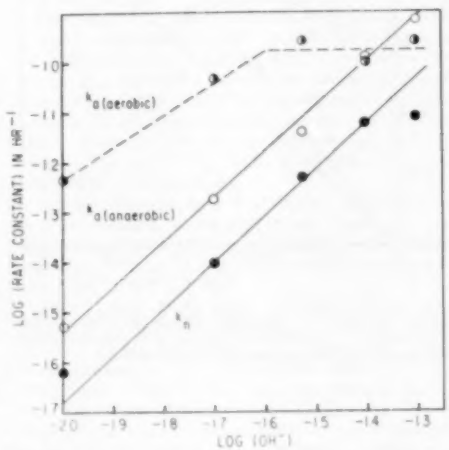

Fig. 5.—A plot showing the hydroxide-ion dependency of the reactions involved in the base catalyzed degradation of prednisolone.

$$k_{a(aerobic)} = \frac{b(OH^-)}{a + c(OH^-)} \qquad \text{(Eq. 15)}$$

where a, b, and c are arbitrary constants. When (OH^-) is small then, $a > c(OH^-)$ and

$$k_{a(aerobic)} \text{ approaches } \frac{b(OH^-)}{a} \qquad \text{(Eq. 16)}$$

and the hydroxide-ion order is one. When (OH^-) is large then, $c(OH^-) > a$, and

$$k_{a(aerobic)} \text{ approaches } \frac{b}{c} \qquad \text{(Eq. 17)}$$

and here the reaction would be zero-order with respect to hydroxide-ion. At intermediate concentrations the order would be between one and zero. While the present limited data indicate this behavior, additional data are required to substantiate this hypothesis.

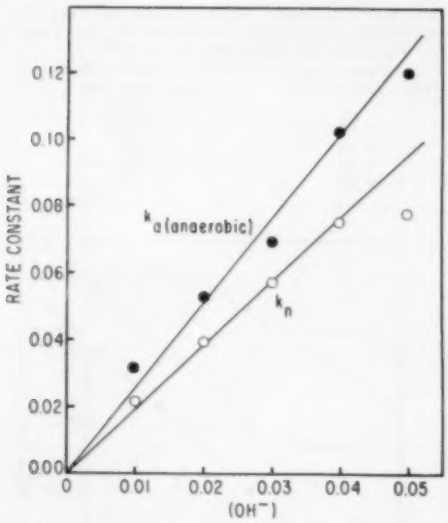

Fig. 6.—A plot showing the hydroxide-ion dependencies of the reactions involved in the anaerobic base-catalyzed degradation of prednisolone.

Figure 6 illustrates the linear dependencies of $k_{a(anaerobic)}$ and k_n on the hydroxide-ion concentration. The specific hydroxide-ion catalytic constants for the reactions described by $k_{a(anaerobic)}$ and k_n were approximated from this plot to be 2.5 liter mole^{-1} sec.$^{-1}$ and 1.9 liter mole^{-1} sec.$^{-1}$, respectively.

The complex nature of steroid degradation suggested by other workers using classical methods of separation and identification is essentially confirmed by the results of this kinetic study. At least three hydroxide-ion catalyzed competitive reactions, occurring at measurable rates, are responsible for the loss of the dihydroxyacetone function of prednisolone. Two or more oxygen-independent reactions form neutral and acidic steroidal products while at least one oxygen-dependent reaction yields an acidic product.

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The Pharmacology of Two Structurally Isomeric Mono-Phenyl Substituted 1,2,4-Triazoles*

By W. R. GIBSON, E. E. SWANSON, and D. B. MEYERS

Pharmacological comparison of 1-phenyl-1,2,4-triazole (1–P) and 4-phenyl-1,2,4-triazole (4–P) revealed that these two-position isomers produce qualitatively opposite effects. In studies performed on intact unanesthetized mice and rats, 1–P caused depression and impairment of motor function, whereas 4–P caused excitation and clonic convulsions. Further experiments on anesthetized and spinal cats and on isolated tissue preparations suggested that both agents act at brain-stem level, and that neither triazole produces any direct peripheral autonomic effect. Investigation of interaction between isomers indicated that either compound was capable of antagonizing the action of the other.

DIFFERENCES observed in pharmocological actions of chemicals of similar structure are usually quantitative rather then qualitative. Infrequently, however, it is possible to demonstrate that a member of a homologous series of chemicals exerts an action that is qualitatively opposed to the actions of other members. Such aberrancy was observed by Swanson and Chen (1) in the action of 1,3-dimethylbutyl-ethyl-barbituric acid, and by Slater, *et al.* (2), who described the convulsant action of 2,2-diethyl-1,3-butanediol, a homolog of the depressant 2,2-diethyl-1,3-propanediol.

Qualitatively opposed action of position isomers was described by Gross and Featherstone (3), who found that the mildly stimulant effect of 1-phenyl-5-methyl tetrazole was changed to a weak depressant action when the position of the phenyl and methyl groups was reversed. McNamara and Krop (4) have reported that among the stereoisomeric hexachlorocyclohexanes, the gamma configuration is stimulant, whereas the beta and delta isomers are depressant.

In 1901, Pellizzari, *et al.* (5), reported that

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4-phenyl-1,2,4-triazole (4–P) produced convulsions in frogs and dogs. Our own observation that the position isomer of this compound, 1-phenyl-1,2,4-triazole (1–P) possessed depressant properties served as a stimulus for a more extensive investigation of the two isomers.

EXPERIMENTAL AND RESULTS

From preliminary evidence that the triazoles exerted predominantly central nervous system (CNS) effects, emphasis was placed upon the evaluation of this activity; however, the possibility that they might exert autonomic or myoneural activity was also explored. Several species of animals were used in this study, including Standard-strain albino mice, Wistar-strain albino rats, rabbits, guinea pigs, and cats. The triazole solutions administered were prepared by dissolving the hydrochloride salt in water and neutralizing with sodium bicarbonate to a pH of 5.5. Concentrations varied from 0.1 to 10.0% so that the volume of solution administered was appropriate for the test.

Median Anesthetic, Convulsive, and Lethal Doses.—1–P was administered i. v. as a 2.0% solution to rats weighing 110 to 140 Gm., and as a 1.0% solution to mice weighing 18 to 22 Gm. to determine the median anesthetic (AD_{50}), and median lethal doses (LD_{50}) and their standard errors (S. E.). Dose response curves were obtained from which the medians were computed by the method of Bliss (6). It should be noted that the term "anesthetic" is not intended to imply that 1–P produces a useful anesthesia, but rather that it is used to denote a specific level of depression. The criterion for attainment of this level was absence of animal response to painful stimulation. 1–P abolished this response for only very brief intervals of one to five minutes, after which the animal rapidly recovered from the maximal depression to a quiescent, semicomatose state that persisted for four to six hours. It was found that the $AD_{50} \pm$ S. E. of 1–P in mice was 157.9 ± 26.84 mg./Kg., and in rats was 134.0 ± 6.03 mg./Kg.

Animals used in LD_{50} studies were observed for twenty-four hours, and it was found that all animals for which 1–P was lethal died within the first three hours, apparently from respiratory failure. The $LD_{50} \pm$ S. E. of 1–P in mice was 261.0 ± 15.7 mg./Kg. and in rats was 155.0 ± 6.2 mg./Kg.

The intravenous median convulsive dose (CD_{50}) and LD_{50} of 4–P were similarly obtained in rats and mice. The triazole was administered as a 0.5% solution to mice and as a 1.0% solution to rats. A convulsion was considered an episode of at least five seconds of facial clonus accompanied by myoclonic jerks. Tremulousness and excitation were not considered convulsive phenomena. Doses slightly in excess of the computed CD_{50} occasionally caused tonic-extensor convulsions after initial clonic convulsions. In the lethal range of doses, tonic-extension usually occurred and mice often died during this crisis. Most animals, however, died in post-ictal depression.

The $CD_{50} \pm$ S. E. of 4–P in mice was found to be 27.24 ± 1.36 mg./Kg. and in rats, 17.42 ± 0.61 mg./Kg., and the $LD_{50} \pm$ S. E. in the same species was 41.19 ± 1.44 mg./Kg. and 37.96 ± 2.35 mg./Kg. respectively.

Oral Activity.—Both triazoles were administered orally to rats weighing 100 to 130 Gm. The concentrations of the solutions were adjusted so that no animal received a volume in excess of 1.5 ml. Using the criteria described for anesthesia and convulsions in the preceding paragraph, it was found that 1–P did not produce anesthesia except at lethal doses. The oral $LD_{50} \pm$ S. E. of this compound was 673.6 ± 43.8 mg./Kg.

On the other hand, 4–P produced convulsions in rats at sublethal doses, and it was found that the oral $CD_{50} \pm$ S. E. of this chemical was 99.3 ± 4.6 mg./Kg. The oral $LD_{50} \pm$ S. E. in the same species was 133.7 ± 9.1 mg./Kg.

Effect upon Barbiturate Sleeping Time.—Prolongation of hexobarbital sleeping time is a useful method for detection of subtle depression that is often inadequate to produce detectable objective symptoms. Since 1–P displayed depressant properties, an experiment was designed to show the effects of the triazoles when they were administered either before or after the barbiturate. Fifty mice weighing 18 to 22 Gm. were used, and these were divided into five groups of ten animals each. The first group received 75 mg./Kg. of 1–P subcutaneously as a 0.75% solution, and by the same route, group two received 15 mg./Kg. of 4–P as a 0.15% solution. Thirty minutes later all five groups were injected intraperitoneally with 100 mg./Kg. of 1.0% hexobarbital. Five minutes after hexobarbital administration, groups four and five were injected intraperitoneally with 75 mg./Kg. of 1–P and 15 mg./Kg. of 4–P respectively. Group three was maintained as a control group.

Following this treatment, all animals exhibited a loss of righting reflex and were placed on their backs. The sleeping time of each animal was then measured as the time that elapsed between injection of barbiturate and return of righting reflex. Each sleeping time was converted to its logarithm, and the geometric mean sleeping time and its standard error were calculated for each group. These data, which show the prolonging action of 1–P and the antagonistic effect of 4–P, are shown in Table I.

Effect upon Electrical Convulsion Threshold.—The method employed in this study was a modification of a method reported by Bogue and Carrington

TABLE I.—TRIAZOLE EFFECT UPON DURATION OF HEXOBARBITAL SLEEP

Drug	Dose, mg./Kg.	Route	Geo. Mean Sleeping Time ± S. E. in Min.	Per Cent of Control ± S. E.
1–P	75.0	S. c.	217.4 ± 13.52	732.7 ± 107.0
4–P	15.0	S. c.	21.7 ± 3.9	73.0 ± 16.3
Control (Hexobarbital only)			29.7 ± 3.9	100.0
1–P	75.0	I. p.	137.5 ± 24.1	463.4 ± 101.7
4–P	15.0	I. p.	16.8 ± 3.8	56.7 ± 14.7

(7). The apparatus required was an electrical stimulator described by Woodbury and Davenport (8) and modified by the introduction of a switch that permitted continuous application of electrical stimulus. Through such a modification, it was possible to determine the threshold of tonic-extensor convulsions in terms of the number of ma.-seconds required to elicit such convulsions.

Rats weighing 90 to 120 Gm. were chosen for test animals. The convulsive threshold was established for each animal by measuring the time required to elicit tonic-extension when the animal was shocked with 7.5 ma. of current by means of ear-clip electrodes. Total time multiplied by current intensity was convulsion threshold in ma.-seconds, and no animal was used in this study whose threshold was below 18.75 or above 37.5 ma.-seconds. Sixty animals were selected twenty-four hours before the test, and thresholds determined on the first trial were considered to be normal thresholds.

The sixty rats were divided into six groups of ten animals each. Groups one and four received 25 mg./Kg. of 1–P i. v. as a 0.5% solution. Groups two and five received 10 mg./Kg. of 4–P i. v. as a 0.2% solution. Groups three and six were maintained as untreated controls. The convulsion thresholds of the animals in groups one, two, and three were determined at five, ninety, and three hundred minutes after drug administration, and of groups four, five, and six at thirty and one hundred eighty minutes. By such a method it was possible to show the effect of the triazoles upon convulsion threshold for a period of five hours.

Variances in threshold were common, even among untreated animals; however, individual variance was minimized when the average of each group was computed. This was accomplished by determining the arithmetic mean threshold of each group at the different times studied, and as seen in Fig. 1, the means are indicative of a trend of activity of the two compounds.

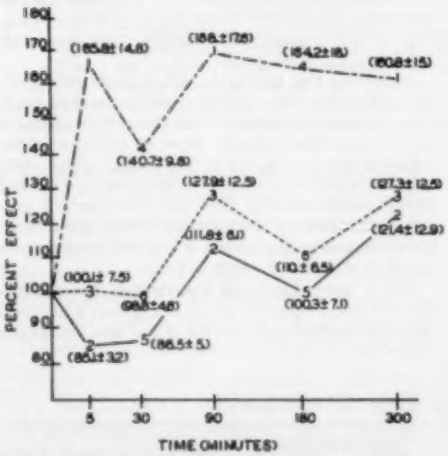

Fig. 1.—Electrical convulsion thresholds of rats represented as percent increase or decrease of normal threshold. Groups 1 and 4 (—— – —— –) received 25 mg./Kg. of 1–P i.v. Groups 2 and 5 (———) received 10 mg./Kg. of 4–P i.v. Groups 3 and 6 (———) are untreated controls.

Figure 1 shows that 1–P was quite effective in raising convulsion threshold; whereas 4–P increased animal susceptibility to convulsions. The latter effect was less dramatic than the former. Repeated stimulation of the animals within a relatively short time resulted in increased threshold, even in the control group.

Blood Pressure, Nictitating Membrane, and Ipsilateral Flexor Reflex Studies in Cats.—These studies were made to investigate systemic actions that were not apparent in intact, unanesthetized animals employed in the above experiments. Cats were first etherized, necessary body areas shaved, and polyethylene tubing inserted into the saphenous vein. Through this tubing all intravenous injections were made. The first injection was 50 mg./Kg. of chloralose in a polyethylene glycol vehicle, after which ether inhalation anesthesia was stopped.

The trachea was cannulated, and both common carotid arteries and the right cervical sympathetic chain were isolated. The right posterior tibial nerve, and the tendinous insertion of the right anterior tibialis muscle were exposed and dissected free of surrounding tissue. The muscular branch of the right sciatic and the right femoral nerves were isolated and severed. The right femur was exposed midway between its articulations and a clamp affixed to the exposed bone surface which immobilized the right leg. Blood pressure was recorded from the left femoral artery in the usual manner.

Right nictitating membrane response was recorded by a lever attached to the membrane by a thread. Contraction of the nictitating membrane resulted when stimulation of 10 volts of 0.2 millisecond duration at 10 stimuli per second was delivered to the right cervical sympathetic chain by means of shielded electrodes. Right anterior tibialis muscle response was recorded by a spring-loaded lever which was attached to the tendon of the muscle by a thread. Muscle contraction was caused by stimulation of the right posterior tibial nerve to which were attached shielded electrodes. These were activated by delivery of 10 volts of 0.2 millisecond duration at 6 stimuli per minute.

Typical responses were routinely produced in these animals by epinephrine, methacholine, and histamine. These were administered before and after triazole administration to determine possible triazole influence upon their effects. Representative tracings from such studies are shown in Figs. 2 and 3.

It was found that neither 20 mg./Kg. of 1–P nor 5 mg./Kg. of 4–P altered carotid sinus reflex, contraction of nictitating membrane, or histamine and methacholine responses. The dose of 4–P administered had practically no effect upon blood pressure, but the dose of 1–P caused a near vascular collapse. There was a suggestion that both triazoles influenced epinephrine response, the 4–P accentuated, and the 1–P depressed.

The flexor reflex was the response most affected by triazole administration. 1–P abolished spontaneous contractions, and reduced amplitude of muscle contraction to nerve stimulation. These effects suggested a depression of some component in the reflex pathway. 4–P however, caused the opposite effect, and administration of this triazole elicited immediate, spasmodic contractions of the muscle sug-

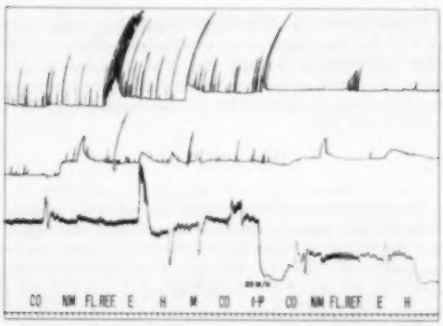

Fig. 2.—Male cat, 2.7 Kg., chloralose anesthesia. Top line is the response of the anterior tibialis muscle to reflex stimulation. The second line is the nictitating membrane response, and the third line is blood pressure. The bottom line is time in minutes. CO (carotid occlusion); NM (stimulation of the nictitating membrane); Fl Ref (reflex stimulation of the anterior tibialis muscle); E (epinephrine, 0.005 mg./Kg.); H (histamine, 0.005 mg./Kg.); M (methacholine, 0.001 mg./Kg.); and 1–P (1-phenyl-1,2,4-triazole).

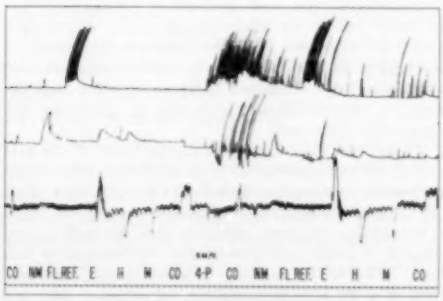

Fig. 3.—Female cat, 2.5 Kg., chloralose anesthesia. Top line is the contractions of the anterior tibialis muscle. Second line is response of nictitating membrane, and third line is blood pressure. The bottom line is time in minutes. CO (carotid occlusion); NM (stimulation of nictitating membrane); Fl Ref (reflex stimulation of anterior tibialis muscle); E (epinephrine, 0.005 mg./Kg.); H (histamine, 0.005 mg./Kg.); M (methacholine, 0.001 mg./Kg.); 4–P (4-phenyl-1,2,4-triazole).

gestive of myoclonic convulsions. The flexor reflex was later shown to be hyperactive. These observations indicated increased irritability within the neural pathway.

Triazole Action in Spinal Cats.—Cats with high cervical spinal cord transection were used in an attempt to learn if the effects observed in the anesthetized animal were of cord origin, or if they resulted from effects upon higher centers. Animal preparation was identical to that of the anesthetized cats, with the exception of cord transection at the second cervical vertebra, and subsequent need of artificial respiration maintained with a Starling pump. Primary interest in this preparation was the effect of the triazoles on the flexor reflex. Epinephrine responses were not altered as much in spinal cats as they were in cats with intact cerebro-spinal axis. Other responses were essentially the same. The dose of 4–P was the same as previously administered but the dose of 1–P was reduced to 15 mg./Kg. to avoid the precarious fall in blood pressure.

Pertinent observations that resulted from these experiments were failure of either triazole to influence flexor reflex, which indicated absence of direct cord effect. Representative tracings are those shown in Figs. 4 and 5.

Isolated Tissue Studies.—These experiments were performed on guinea-pig and rabbit intestinal strips and on rat uterus. The tissues were bathed in a 100-cc. chamber of aerated Locke-Ringer's solution which was maintained at 37°. The guinea-pig intestinal strips were treated with histamine and methacholine, and the rabbit intestinal strips with

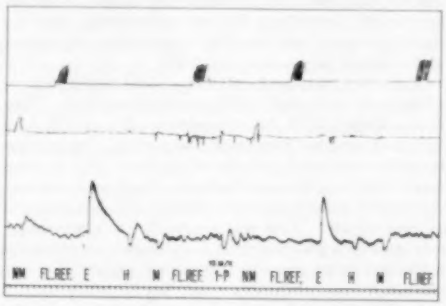

Fig. 4.—Female cat, 3.2 Kg., C-2 spinal cord transection. Top line is the contractions of the anterior tibialis muscle. Second line is nictitating membrane, and third line is blood pressure. Bottom line is time in minutes. NM (stimulation of nictitating membrane); Fl Ref (reflex stimulation of the anterior tibialis muscle); E (epinephrine, 0.005 mg./Kg.); H (histamine, 0.005 mg./Kg.); M (methacholine, 0.001 mg./Kg.); 1–P (1-phenyl-1,2,4-triazole).

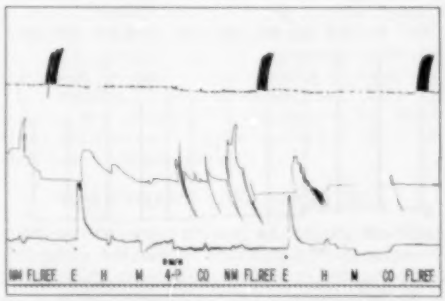

Fig. 5.—Female cat, 2.8 Kg., C-2 spinal cord transection. Top line is contractions of the anterior tibialis muscle. Second line is the nictitating membrane response, and the third line is blood pressure. Bottom line is time in minutes. CO (carotid occlusion); NM (nictitating membrane stimulation); Fl Ref (reflex stimulation of the anterior tibialis muscle); E (epinephrine, 0.005 mg./Kg.); H (histamine, 0.005 mg./Kg.); M (methacholine, 0.001 mg./Kg.); 4–P ¡ (4-phenyl-1,2,4-triazole).

epinephrine and posterior pituitary extract. Ten mg. of 4–P was without effect on any tissue or drug response. Ten mg. of 1–P failed to alter histamine, methacholine, or epinephrine responses, but it did partially antagonize the musculotropic stimulant effect of 0.01 mg. of posterior pituitary extract. It abolished spontaneous, rhythmic contractions of rat uterus. Lower doses of this triazole were less active, and 5.0-mg. doses were virtually without effect. It seems unlikely that these studies indicated either peripheral autonomic effect or musculotropic action, for the concentration of 1–P required to elicit relaxation was greater than could be expected to exist at these tissues *in situ*, even if selective distribution were postulated.

Triazole Interaction.—Available experimental evidence suggested that the triazoles had similar sites of action, with opposite effects upon these sites. It was thought that the action of one might be antagonized by the other, and an experiment was designed to learn whether the respiratory depression of 1–P could be reversed by 4–P.

This study was performed on albino rabbits whose respiratory rate and volume were measured. This was accomplished by connecting the rabbit, by means of a rubber face mask and tubing, to a spirometer that recorded volume of expired air. A two-way valve was located between mask and spirometer which permitted entry of fresh air, but trapped all expired air. This was conducted to the spirometer, to which was attached a stylus that recorded on a smoked drum the amount of air expired for one minute.

In Fig. 6 may be seen the results of this experiment. Intravenous administration of 37.5 mg./Kg. of 1–P caused a decrease in both respiratory rate and volume. After fifteen minutes of this depression, 5.0 mg./Kg. of 4–P was administered intravenously. For the next twenty minutes both respiratory rate and volume were increased, but volume then decreased and attained a level below that caused by 1–P alone.

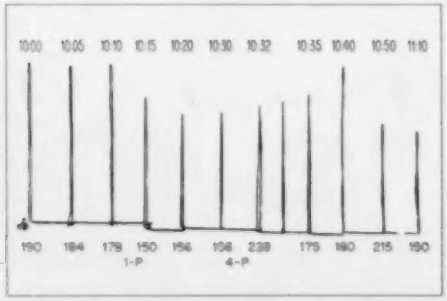

Fig. 6.—The respiratory stimulant effect of 4-phenyl-1,2,4-triazole (4–P) on respiratory depression produced by 1-phenyl-1,2,4-triazole (1–P) in the intact, unanesthetized rabbit. Vertical lines represent respiratory volume, the numbers under the vertical lines represent respiratory rate, and the time of each measurement is indicated above the vertical lines.

These observations indicated that the antagonism of 4–P for the respiratory depressant effect of 1–P was transient, and that the post-stimulation depression of the stimulant triazole acted to intensify the depression produced by the depressant triazole.

DISCUSSION

Experiments performed with the phenyl-triazoles have shown these isomers to be qualitatively opposite in action. There is little positive evidence to suggest that these compounds act on other than the CNS, for effects of 1–P observed on isolated smooth muscle could result from musculotropic toxic actions.

From available evidence it is impossible to identify conclusively the primary site of action of either isomer. It is quite apparent, however, that the actions of the triazoles on the spinal cord are secondary to actions on higher centers. This was shown by the inability of either compound to influence the ipsilateral flexor reflex of cats with high spinal cord transection; whereas, both isomers profoundly affected the same reflex in cats with intact cerebrospinal axis. It is likely that the triazoles act on the brain stem, and it is possible that the medulla can be excluded as a primary target site because neither compound demonstrated the profound blood pressure or respiratory effects at effective, nontoxic doses that could be expected if either had a predominantly medullary locus of action. In another respect, the stimulant 4–P produces a pattern of action that resembles pentamethylenetetrazole, a primarily diencephalic stimulant.

From reported physical characteristics of the triazoles, an explanation of their opposite effects may be found. Jensen and Friediger (9) and Orgel, *et al.* (10), have determined the dipole moments of these compounds, and these indicate that the convulsant triazole is more hydrophilic than its depressant isomer. This would indicate greater lipophilic attraction of 1–P, and it could act to increase neurone stability by increasing the permeability of nerve cell membrane. It is possible that the hydrophilic 4–P acts to decrease neurone stability through a decrease in membrane permeability, a mechanism proposed by Cicardo, *et al.* (11), for this type of agent.

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A Thermistor Thermometer and Direct-Reading Isotonicity Meter*

By FRANK M. GOYAN

A differential thermistor thermometer intended for measuring freezing-point depressions is described. The instrument provides one temperature range (0–1°) and two isotonicity ranges (0–1% NaCl and 1–2% NaCl) by means of a selector switch on the front panel. The principle of the linear isotonicity scale and methods for its calibration and use were presented in an earlier paper.

BALLARD AND GOYAN (1) have shown that it is entirely practicable to establish a linear isotonicity scale based upon freezing-point lowering or other colligative property related to ideal semipermeable membranes. It is hoped that isotonicity values in per cent NaCl may be used without confusion as a measure of colligative properties of solutions, regardless of intended biological applications. This usage need not prejudice the biological meaning of the concepts when qualified by referring to specific biological systems. In other words, there should be no incongruity in the statement that a certain solution of urea known to have an isotonicity value of 0.9 per cent NaCl fails to protect certain red cells from undergoing hemolysis. Whenever the meaning is not clear from the context, it may be desirable to employ an abbreviation indicating the method of measurement used. For example, 0.9 per cent NaCl (f. p.) would represent an isotonicity value derived from freezing-point measurements.

CIRCUIT DETAILS

Regardless of the acceptance of the term isotonicity in the sense indicated above, a differential thermometer capable of being easily calibrated to read directly any preselected linear function of freezing-point depressions is of considerable value. Resistors 11 and 12 of Fig. 1 determine the ratio required (2). In calculating the values for an isotonicity scale, resistor 11 is taken arbitrarily at 10 megohms and resistor 12 is calculated by the equation $(0.53/0.90) = 10/(10 + x)$ where x is the value in megohms sought. Selection from 10 meg. and 6.8 meg. resistors gave a satisfactory pair, although the solution suggested above gives 6.98 meg. Obviously, the same type of arithmetic can be applied to any desired ratio. Loading factors, although slight, indicate the need for final check and adjustment.

The bridge circuit is shown in Fig. 1 directly above the word "bridge." Resistor 5 is a potentiometer that supplies power to the bridge in such a way that it is easy to achieve balance for widely different values of resistor 7 without producing large changes in temperature sensitivity. Any bridge may be considered to be made up of two potentiometers in parallel. Off-balance conditions are obtained when the potential at one center tap differs from the potential at the other. Satisfactory linear response of this off-balance potential is obtained by selecting a suitable value for resistor 7, following the work of Zeffert and Witherspoon (3). Values for 7 ranging from the resistance of the thermistor to three times this value gave linear temperature response within ±0.01° for a 1° range. The value shown in Fig. 1 produced a linear response well within this limit.

The development of the vacuum-tube voltmeter circuit followed conventional lines. Some of the features of the twin-triode, vacuum-tube, bridge circuit were suggested by the circuit used in the RCA Senior VoltOhmyst (2). Circuit constants were adjusted to give a sensitivity of the order of magnitude of 200 mv. for full-scale deflection.

The selector switch designated X, Y, and Z in Fig. 1 is a typical 3-deck, 5-position switch shown in position 4 which establishes the 0–1% isotonicity range. Position 5 directly below in the circuit diagram differs only in that the voltage is read from a different point on the bridge. This position establishes the 1–2% isotonicity range when resistor 8 is adjusted to equal the change in resistance of the thermistor corresponding to a 1% change in isotonicity. Switch position 3 directly above 4 in Fig. 1 differs from 4 only by a change in the voltage range of the V. T. V. M. This position is used for adjustment at the ice point and for reading freezing-point depression in ° C. Position 2 is used to balance the V. T. V. M. initially with resistor 19 and daily with 18. Position 1 provides for the use of the basic V. T. V. M. circuit as an independent unit.

ADJUSTMENT AND OPERATION

The principles involved in establishing the linear isotonicity scale and in providing suitable reference points on the temperature scale have been presented in an earlier publication (1). The construction of the thermistor probe and certain techniques established for its use have also been described in the same paper. Calibration and use of this instrument requires two fixed temperatures, one at the ice point and one reading well beyond the half-way point on the 0–1° freezing-point depression scale.

Adjustment is begun with the thermistor probe in the ice bath. Switch to position 2, turn the power on, and allow the instrument to warm up for a few minutes. Adjust resistor 19 until the meter reads

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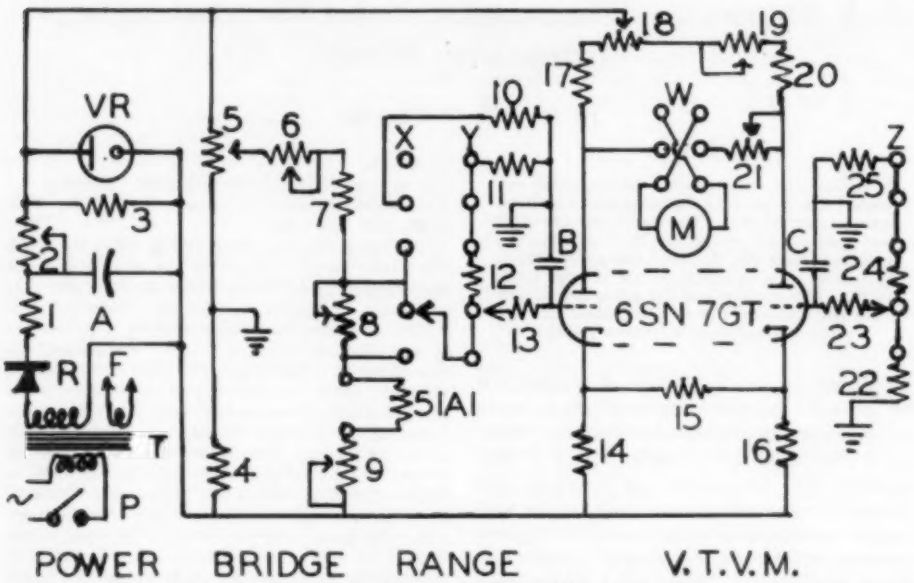

Fig. 1.—Circuit diagram: VR—voltage regulator tube OA 3, M—0–100 μ A. D. C. meter; R—selenium rectifier; T—power transformer; F—filament winding. Capacitors: A—40 mfd. electrolytic; B and C—0.001 mfd. mica. Switches: P—power switch; XYZ—ganged rotary selector switch; W—rotary D. P. D. T. Resistors: 1—560 ohms, 1 watt; 2—5 K, 50 watt; 3—270 K; 4—2.7 K; 5—10 K pot.; 6—75 K pot.; 7—1 meg., 1 watt; 8—10 K pot.; 9—5 K pot.; 10—330 K; 11—10 meg.; 12—6.8 + meg. (see text); 13—2.7 meg.; 14—15 K, 1 watt; 15—270 ohms, 1 watt; 16—15 K, 1 watt; 17—5 K, 1 watt; 18—500 ohm pot.; 19—40 K pot.; 20—2.7 K; 21—10 K pot.; 22—330 K; 23—2.7 meg.; 24—6.8 meg.; 25—10 meg.; 51A1—Type 51A1 thermistor made by the Victory Engineering Corporation of Union, N.J.

approximately zero while 18 on the front panel is in a central position. Complete the V. T. V. M. zero adjustment with 18 and switch to position 3. Balance the bridge with the aid of resistors 5 and 6 while 9 on the front panel is centrally positioned. Bridge balance is indicated by a zero reading of the meter. The ice bath must be stirred.

Place the thermistor in the lower reference temperature bath and stir until a constant reading is obtained, using reversal switch W if the meter is off scale. Cause the meter to read the known temperature of the reference bath by adjusting resistor 21.

Rinse or wipe the thermistor probe and return it to the ice bath. Check, and readjust if necessary, the V. T. V. M. zero. With the aid of resistor 9 on the front panel, cause the meter to show any convenient reading between 0.35 and 0.55°. Switch to position 4 and verify the conversion from freezing-point depression to isotonicity, making adjustments with the aid of a 1 meg. variable resistor temporarily soldered in series with resistor 11 or 12. Verify the conversion and the V. T. V. M. zero before replacing the variable resistor with a fixed resistor of about the same value. Prepare a separate ice bath, substituting dilute NaCl for water, if the range of resistor 9 is inadequate for a final check covering nearly the full scale.

While the thermistor probe is still in the ice bath and resistor 9 is adjusted to give a convenient reading, switch from position 4 to position 5 and reverse meter polarity with switch W. Adjust resistor 8 until the sum of the two readings is unity. Verify the adjustment of resistor 21 with the aid of the additional reference temperature before completing this adjustment.

Only resistors 9 and 18 need daily adjustment. For accurate work, it is wise to check the adjustment of resistor 21 with the aid of the reference temperature bath from time to time. Inexperienced operators must be warned to be assured that the probe has reached equilibrium temperature with stirring and that the meter is fully warmed up before making adjustments. On the instrument in use by the author, the selector switch positions are labeled, "V. T. V. M.," "Zero," "Temp., ° C.," "% NaCl," "Add One." Resistor 9 is labeled "Ice Point Zero" and 18 is labeled "V. T. V. M. Zero." These two controls are all that are needed on the front panel. Resistor 21 is available from the back and is labeled "Sensitivity."

RESULTS

The author's instrument has been used as a development model and as a teaching instrument for some time. It has given adequate results in the hands of students for over a year. After some experience, students are able to obtain freezing-point depression values for solutions of NaCl which agree with theoretical within 0.01°.

Using a saturated solution of boric acid in equilibrium with ice as a reference temperature, sensitivity is checked frequently and often found to require no adjustment. The front panel controls have

TABLE I

Temperature, °C. (Mercury Thermometer)	Thermistor Temp., °C.	Thermometer Isotonicity, % NaCl	Isotonicity (Calculated), % NaCl
0.000	0.00	0.00	0.00
0.117	0.12	0.20	0.20
0.264	0.26	0.44	0.44
0.392	0.40	0.68	0.68
0.525	0.53	0.90	0.90
0.665	0.67	1.14	1.14
0.911	0.92	1.58	1.56
0.977	0.98	1.70	1.66

recently been changed to the larger resistance values shown in Fig. 1 in order to cover variations found in practice.

Table I shows the results of a series of measurements made with the aid of a decade resistor box which was set to values previously determined with considerable care to represent the resistance of a thermistor probe at the temperatures listed in the table. The results indicate satisfactory performance within the range usually required. The conversion to isotonicity could be improved in the higher region by more careful adjustment of resistor values.

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Studies on Antacids VI. Adsorption Effects of Various Aluminum Antacids upon Some Simultaneously Administered Synthetic Anticholinergic Drugs*

By I. W. GROTE and MARIBELLE WOODS

A study was made of the possible adsorption of various synthetic drugs often given simultaneously with aluminum compounds in the treatment of gastric hyperacidity. The approximate LD_{50} for each drug upon oral administration to mice was found, and the same dose was then given in admixture with various aluminum preparations to determine if the same LD was obtained. One of the drugs tested was adsorbed by aluminum hydroxide gel, while another aluminum antacid did not cause adsorption to the same extent, if at all.

THIS PAPER is a continuation of our work (1) in which we showed the adsorptive effects of various aluminum antacids upon simultaneously administered anticholinergic drugs. The present study covers some of the newer synthetic anticholinergic drugs. Like the alkaloids of the belladonna group, these synthetic drugs are often used along with aluminum antacids for their therapeutic action in the more rapid control of peptic ulcer pain. Since some of the newer drugs are similar in chemical constitution to the naturally occurring alkaloids, it seemed reasonable to assume that they too might be adsorbed by some of the aluminum antacids.

METHOD

The method of test is the same as that used in the previous paper. It consists of finding the approximate mouse oral LD_{50} for the drug to be tested, and then giving this same dose in admixture with the aluminum preparation. The LD_{50} dosage was selected in place of the usual LD_{50} in order to have a wider margin in which to show adsorption of the anticholinergic drug. The adsorption can take place either in the formation of a substance nonrecoverable in the mouse intestinal tract, or in one recoverable so slowly that the toxic effect of the drug is greatly reduced. Presumably this would indicate to some extent whether the desired therapeutic effect in the human patient would be achieved if the two drugs were administered simultaneously. Again, as Seifter, *et al.* (2), has pointed out, such a study does not permit the use of ratios of aluminum antacids to synthetic anticholinergic drugs which even approximate those actually used in human therapy. However, this factor should merely minimize the adsorption effect which would be found if the actual human therapeutic ratio was used, since the dose of aluminum antacid would be in greater ratio, and thus more adsorptive.

As in the previous study, we used the maximum dose of the aluminum compounds which can be conveniently given orally to a mouse by stomach tube, namely 200 mg. in a volume of 1.0 cc. This large dose of antacid completely neutralizes the mouse stomach acidity, and the drug-antacid mixture passes through the intestinal tract in a viscous mass. However, the fact that some drugs demonstrate un-

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TABLE I.—ACUTE ORAL TOXICITY OF ANTICHOLINERGIC DRUGS IN MIXTURE WITH ALUMINUM ANTACIDS

Anticholinergic Drug	Dose[a] mg.	Mortality[b] Anticholinergic Drug Alone	Anticholinergic Drug in Mixture with Dihydroxyaluminum aminoacetate, 200 mg.	Anticholinergic Drug in Mixture with $Al(OH)_3$ Gel, Dried, 200 mg.
Pro-Banthine	21	8/8 (100%)		
	19.5	22/24 (92%)	17/24 (71%)	16/24 (67%)
	18	9/17 (53%)		
Pathilon	37.5	2/2 (100%)		
	30	20/24 (83%)	16/24 (67%)	3/24 (13%)
	25	3/8 (37%)		

[a] Dose in mg. active principle of commercial tablets.
[b] Mortality is expressed as number of female mice (24 ± 2 Gm.) dead in twenty-four hours/number of mice tested.

changed toxicity when given in admixture with the antacid shows that the method is valid in principle.

Female mice weighing 24 ± 2 Gm. were used for the study. This permitted the use of a single weight of drug-antacid within a series, rather than one based on a mg./Kg. dose. The mixture to be tested was given orally using a syringe fitted with a blunt No. 18 needle. Animals were then observed for twenty-four hours and the deaths which occurred during this period were recorded. Twenty-four mice were used for each dosage level.

The antacids tested consisted of a highly active commercially available dried alumina gel, U. S. P. and dihydroxyaluminum aminoacetate, N. F. (powder). Suspensions were prepared containing 200 mg. of antacid plus the desired amount of anticholinergic drug for each 1.0 cc. dose. Because the two antacids are used in medicine in approximately equal doses by weight, a direct comparison by weight is justified. We did not use commercially available aluminum hydroxide magmas in this test series since the previous work shows they give essentially the same results as the ones freshly prepared from the highly active dried material, and since they are rarely available in concentrations as great as 200 mg. per ml.

In the previous study, in order to test the effect of time upon the adsorption of the drug by the antacid, tests were made upon antacid-drug mixtures both prepared immediately before oral administration to the mice and those in which the drug-antacid magmas were mixed and allowed to stand for four days before administration. This was not done in the present work, since the results obtained previously show merely an intensification of the results shown for the freshly prepared mixtures.

The drugs tested were β-diisopropylaminoethyl xanthene-9-carboxylate methobromide, brand of propantheline bromide, Searle (Pro-Banthine) and 3-diethylamino-1-cyclohexyl-1-phenyl-1-propanol-ethiodide, brand of tridihexethyl iodide, Lederle (Pathilon). Methscopolamine bromide (Pamine, Upjohn) is commercially available only in tablets containing 2.5 mg. of the active principle. Due to the bulk of the tablet diluent, it was not possible to give more than 15 mg. of active ingredient (6 tablets per mouse) which failed to kill. Since this drug is somewhat closely related to homatropine reported in the earlier paper, we did not extend this study. Similarly reserpine in doses of 5 to 50 mg. per mouse did not produce death within twenty-four hours. The smaller doses of reserpine appeared to have more readily apparent physical effect on the mouse than the large ones. Since the human dose of reserpine is only 0.1 to 1.0 mg. per day, we did not follow this drug further.

The results obtained with Pro-Banthine and with Pathilon are shown in Table I. Examination of the table shows that the freshly prepared magma made of dried "active alumina" hydroxide U. S. P. when mixed with Pathilon, reduces the oral toxicity to mice from an LD_{33} to an LD_{13}, while it changes an LD_{92} for Pro-Banthine to an LD_{67}. Dihydroxyaluminum aminoacetate N. F. under similar conditions changed the LD_{33} of Pathilon to an LD_{67}, and of Pro-Banthine from an LD_{92} to an LD_{71}.

CONCLUSIONS

When measured by the mouse LD_{50} method, neither dihydroxyaluminum aminoacetate nor aluminum hydroxide show marked adsorptive effects on Pro-Banthine when the two are administered simultaneously. However, there is a marked adsorption of Pathilon by aluminum hydroxide magma, and only slight adsorption by dihydroxyaluminum aminoacetate when tested by the same method.

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Some Factors Influencing the Saprophytic Production of Clavine Alkaloids by *Claviceps purpurea**

By V. E. TYLER, Jr.†

A screening of various ergot samples yielded several isolates capable of unequivocal synthesis of clavine alkaloids in saprophytic culture. Of these, a typical agroclavine-producing strain was selected, and the influence on it of various modifications of nutrient media and cultural conditions was investigated. Mannitol and ammonium succinate were confirmed as superior carbon and nitrogen sources in the Stoll-Abe medium, but greatly improved yields of alkaloid were obtained when increased concentrations of both these compounds were employed. Additions of ascorbic acid or biotin and substitution of casein hydrolysate for succinic acid did not increase alkaloid production in this medium. A casein hydrolysate-mannitol-basic nutrient medium proved decidedly inferior for alkaloid production, but a carbohydrate-peptone medium yielded excellent results. Submerged cultures contained considerably lower concentrations of alkaloids than stationary cultures. The importance of an optimum fermentation time for all media under all conditions was established.

A DECADE has elapsed since the monumental announcement by Abe (1) in 1948 of the isolation of a new, water-soluble ergot alkaloid, agroclavine, from the saprophytic culture of a strain of *Claviceps purpurea* (Fries) Tulasne normally parasitizing *Agropyron semicostatum* Nees in Japan. During this time, the production of a number of other new, chemically-related alkaloids by various strains of *Claviceps* in saprophytic culture has been reported (2–6), but no detailed study of the influence of nutritional and cultural factors on the production of these alkaloids has appeared.

Chemically, the compounds have been subjected to structural examination and have been characterized as derivatives of ergoline. Indicative of their relation to agroclavine, they have been assigned names derived by adding suitable prefixes to the clavine stem. For convenience, the whole group may be referred to as clavine alkaloids.

For the production of agroclavine in saprophytic culture, Abe (1) employed a medium containing mannitol and ammonium succinate as the sole sources of carbon and nitrogen. Stoll, *et al.* (3), subsequently reported that their penniclavine-producing *Claviceps* strain obtained from *Pennisetum typhoideum* Rich. produced alkaloids in all the media tested but gave improved yields when sucrose was employed jointly with mannitol as the carbon source. An Indian patent (7) obtained by Sandoz, A. G. has revealed the importance of adequate levels of iron and zinc in the culture medium for alkaloid production.

Since further specific information regarding the influence of the composition of the medium on the biosynthesis of clavine alkaloids has not appeared in the literature, it seemed worth while to undertake an examination of the effect of various nutritional and other environmental factors on the production of clavine alkaloids in saprophytic culture.

EXPERIMENTAL

General Methods.—Isolates of various ergot samples were prepared by cutting small segments from the inner pseudo-parenchyma tissue of sclerotia and surface-sterilizing them by dipping in diluted Lugol's solution followed by rinses in 70% alcohol and sterile distilled water. Mycelial development followed implantation in culture tubes containing slants of a casein hydrolysate-mannitol-basic nutrient medium (8) solidified with 2% agar. Transfers were subsequently made and maintained on additional tubes of the same medium.

Isolates obtained in this manner were tested for their alkaloid-producing ability by transferring them to Roux-type culture flasks containing 125 ml. of Stoll's (3) modification of Abe's nutrient solution (Stoll-Abe medium). Although Abe, *et al.* (9), had previously shown that their cultures reached a maximum agroclavine content after thirty to thirty-five days and this period of time was selected as a convenient one for preliminary studies, evidence was subsequently obtained that the time of maximum alkaloid content varied considerably, depending upon the composition of the medium and the strain of *C. purpurea* employed. Unless otherwise stated, the cultures were allowed to develop at room temperature for thirty-five days.

In confirmation of the report of Stoll, *et al.* (3), it was found that the quantity of alkaloids produced by any given strain was not exactly reproducible in repetitive experiments. Results were found to vary slightly according to the rapidity of development of the organism, as affected by the amount of inoculum employed, the temperature, and other sim-

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ilar factors. Thus, when possible, the cultures in comparative experiments were inoculated from the same stock culture and allowed to develop simultaneously. At the end of the selected fermentation period, the flasks were harvested and the mycelium separated from the culture medium by straining.

Two milliliters, or an appropriate quantity diluted to 2 ml. of the centrifuged medium, were tested directly for alkaloids by mixing with 4 ml. of *p*-dimethylaminobenzaldehyde reagent (10). After illuminating the mixture for seven minutes with a mercury vapor lamp (11), the solution was transferred to a 1/2 inch test tube and the absorbance determined in a Bausch and Lomb Spectronic 20 spectrophotometer at 590 mμ.

The mycelium was dried in a mechanical convection oven at 45° for forty-eight hours and weighed. Moisture analyses carried out (11) at random on a number of 1-Gm. samples revealed that mycelium treated in this fashion possessed an average residual moisture content of 7%.

Samples were next defatted and assayed by a modification of the procedure of Silber and Schulze (10). The modification consisted of omitting the pH 8 buffer shakeouts and using only the tartaric acid solution for this purpose. In this way, the total alkaloids, both water-soluble and water-insoluble, were present in the combined tartaric acid shakeouts which were assayed in the manner just described for the medium.

Alkaloid concentrations were determined from a standard curve prepared from ergonovine maleate, U. S. P. Reference Standard, and were calculated and expressed as the percentage of agroclavine (mol. wt. = 238.32) on the basis of the volume of the culture medium or the weight of oven-dried (7% moisture), defatted mycelium.

When desired, the composition of the total alkaloid fraction was established by the paper chromatographic method of Wier (12). The filtered culture medium was acidified with tartaric acid and defatted by shaking out with replicate portions of ether or petroleum ether. It was subsequently rendered alkaline with 10% ammonium hydroxide and extracted with a mixture of chloroform-isopropyl alcohol (3:1) or with pure chloroform. The extract was evaporated in a stream of warm air and the residue redissolved in 3 ml. of a 2% aqueous solution of tartaric acid.

Portions (50–150 μl.) of this concentrated extract of the medium or similar portions of the tartaric acid extract from the assay of the mycelium were spotted on one-inch strips of Whatman No. 1 filter paper which had previously been dipped in McIlvaine's buffer at pH 5 and allowed to air dry. The strips were formed by the ascending technique employing water-saturated *n*-butanol as the wash liquid. Under these conditions the principal clavine alkaloids,[1] which could be detected by the characteristic color developed on spraying with 2% *p*-dimethylaminobenzaldehyde in 1 *N* hydrochloric acid, showed the following average R_f values: agroclavine 0.60, setoclavine 0.44, elymoclavine 0.33, penniclavine 0.22.

Selection of Clavine Alkaloid-Producing Strains—A screening program involving the testing of more than 50 samples of ergot from various parts of the world yielded a limited number of cultures capable of the repeated synthesis of appreciable quantities of clavine alkaloids in saprophytic culture.

The *C. purpurea* isolates which showed unequivocal alkaloid production when cultured and tested by the procedures described above were as follows:

Sample 15.—Ergot of *Agropyron semicostatum*, collected near Kyoto, Japan, July 1956, courtesy of Dr. Haruya Shimada.

Sample 33.—Culture of ergot of *Agropyron semicostatum* (Japanese source), courtesy of Dr. E. Glaz, Budapest, Hungary, 1956.

Sample 47.—Ergot of *Pennisetum typhoideum* (French Equatorial African source), courtesy of Dr. A. E. Schwarting, Storrs, Conn., 1957.

Typical quantitative results of alkaloid production obtained with these strains are recorded in Table I.

One of these strains, 15A, was also tested for alkaloid production in the Stoll-Abe medium modified by the addition of 5% of sucrose, since Stoll, *et al.*, reported increased yields with their *Claviceps* culture in this medium. After thirty-five days fermentation the volume of medium in four flasks averaged 74 ml. and contained only 0.77 mg. % of alkaloid in spite of excellent mycelial development (2.23 Gm.).

The mycelial mats of all of these cultures exhibited the usual variations in form and color. At the end of the incubation period the media from these clavine alkaloid-producing cultures had changed from their original colorless condition to different shades of brown or reddish-brown.

Chromatographic analysis of extracts of the medium and mycelium of different cultures revealed agroclavine, setoclavine, elymoclavine, and small quantities of an unidentified water-insoluble alkaloid as the metabolic products of strain 15. Only agroclavine could be detected in the culture of strain 33, but this may have been due to the relatively small quantity of alkaloidal mixture chromatographed. If not completely absent, the other alkaloids were present in a quantity insufficient to be detected by the methods employed. Strain 47 was found to produce agroclavine, elymoclavine, and penniclavine.

In order to study the influence of various factors on clavine alkaloid production, it seemed advisable to select a culture which appeared typical of those examined. For this purpose, strain 15D was chosen, and the bulk of this investigation was carried out employing this isolate.

The Effect of Various Carbohydrates and the Ammonium Salts of Various Organic Acids on Alkaloid Production.—In order to ascertain the influence of certain single carbohydrates on alkaloid production, a series of cultures was prepared consisting of two flasks each of the Stoll-Abe medium in which sorbitol, glucose, maltose, fructose, sucrose, inositol, galactose, lactose, or dulcitol had been substituted in like quantity for mannitol. After inoculation with strain 15D the cultures were allowed to incubate for thirty-two to thirty-five days, harvested, and the culture medium and mycelium assayed as previously described. The average results of the assays of the duplicate flasks of each carbohydrate substituted are reported in Table II.

[1] Reference samples supplied through the courtesy of Dr. A. Hofmann, Sandoz Ltd., Basle, Switzerland.

TABLE I.—ALKALOID PRODUCTION BY TYPICAL CLAVINE-PRODUCING STRAINS OF *Claviceps*

Strain No.	Volume of Medium, ml.	Alkaloids in Medium, mg. %	Dry Weight of Mycelium, Gm.	Alkaloids in Mycelium, %
15A[a]	88	11.71	2.15	0.153
15B	85	12.85	2.31	0.086
15C	85	4.10	2.47	0.082
15D	80	10.69	1.93	0.047
15E	85	7.56	2.43	0.267
33	78	5.40	2.07	0.044
47	80	7.02	1.77	...

[a] Letter designations indicate isolates from different sclerotia of the same sample.

TABLE II.—THE EFFECT OF THE CARBOHYDRATE SOURCE ON ALKALOID PRODUCTION BY STRAIN 15D

Carbohydrate	Volume of Medium, ml.	Alkaloids in Medium, mg. %	Dry Weight of Mycelium, Gm.	Alkaloids in Mycelium, %
Sorbitol	98	5.18	1.63	...
Glucose	85	2.27	1.59	Trace
Maltose	93	1.96	1.59	0.042
Fructose	92	1.19	1.75	0.040
Sucrose	85	1.18	1.62	0.028
Inositol	105	0.54	0.60	Negative
Galactose	100	0.28	0.77	Negative
Lactose	...	...	Negligible	...
Dulcitol	...	...	Negligible	...

Since none of the carbohydrates substituted gave yields superior to those obtained in the mannitol-containing medium, it was decided to determine if variations in the mannitol concentration had any influence on alkaloid production. Consequently two flasks each of Stoll-Abe medium containing 10, 15, and 20% mannitol were prepared and tested as previously described. In this case, however, the development of the organism proceeded more slowly than in the usual 5% mannitol medium, and the incubation period was extended to a total of forty-five days. Also, since the preponderance of alkaloid in a culture is contained in the medium, the mycelium was not assayed. Average results of this trial are recorded in Table III.

The effect of substituting the ammonium salts of various other organic acids for the succinic acid (ammonium succinate) in the medium was next ascertained. Actually, after addition of the acid, the pH of the Stoll-Abe medium is adjusted to 5.2 with ammonium hydroxide and the acid is converted largely to its ammonium salt. This being understood, hereafter for the sake of brevity these compounds will be referred to as acids, the form in which they were originally added to the medium. A series of duplicate cultures containing like quantities (5.4 Gm./L.) of L-malic, citric, fumaric, glutamic, α-ketoglutaric, itaconic, DL-malic, pyruvic, tartaric, citraconic, oxalacetic, and malonic acids instead of succinic acid, was prepared. These were then treated as previously described, incubated thirty-five to thirty-eight days, and assayed with the average results recorded in Table IV.

TABLE III.—THE EFFECT OF INCREASED MANNITOL CONCENTRATIONS ON ALKALOID PRODUCTION BY STRAIN 15D

Mannitol, %	Volume of Medium, ml.	Alkaloids in Medium, mg. %	Dry Weight of Mycelium, Gm.
10	87	25.84	3.37
15	90	39.60	4.26
20	102	34.02	4.57

In considering the results obtained in these experiments with carbohydrates and acids it was decided to combine an increased concentration of mannitol in the medium with increased concentrations of succinic acid. However, since a medium of this composition departed appreciably from the original formula, it was deemed necessary to consider the factor of fermentation time as well. A series of flasks was prepared containing the Stoll-Abe medium modified to contain 15% mannitol. Duplicate flasks of media containing 2.7, 5.4, 10.8, and 21.6 Gm./L. of succinic acid were employed. Following inoculation with strain 15D, small portions of the culture medium of each flask were withdrawn aseptically on the thirty-fifth and fortieth day of incubation. A final assay of the medium was carried out on the forty-fifth day, concluding the experiment.

Mycelial development took place more rapidly in the cultures containing less succinic acid, but a slower, more profuse development followed in the cultures containing larger amounts. That alkaloid accumulation also tended to follow this pattern will be observed from the results recorded as averages in Table V.

The Influence of Certain Modifications and Changes of the Medium on Alkaloid Production.—Various other additions and modifications of the basic Stoll-Abe medium were tested in an attempt to increase alkaloid production.

Since Kaiser and Thielmann (13) obtained some indications that vitamin C might play a role in alkaloid synthesis by *C. purpurea* in saprophytic cultures, a series of culture flasks containing additions

TABLE IV.—THE EFFECT OF THE ORGANIC ACID SOURCE ON ALKALOID PRODUCTION BY STRAIN 15D

Acid	Volume of Medium, ml.	Alkaloids in Medium, mg. %	Dry Weight of Mycelium, Gm.	Alkaloids in Mycelium, %
Succinic	90	12.32	2.07	0.026
L-Malic	97	10.04	1.71	0.028
Citric	100	9.40	1.55	0.209
Fumaric	91	7.16	1.51	0.104
Glutamic	97	6.92	1.90	0.041
α-Keto-glutaric	85	6.80	1.56	0.013
Itaconic	85	3.32	0.61	0.025
DL-Malic	90	1.65	1.97	0.019
Pyruvic	90	0.81	...	...
Tartaric	95	0.46	1.49	0.203
Citraconic	...	...	Negligible	...
Oxalacetic	...	...	Negligible	...
Malonic	...	...	Negligible	...

TABLE V.—THE EFFECT OF THE SUCCINIC ACID CONCENTRATION, WITH 15% MANNITOL, ON ALKALOID PRODUCTION BY STRAIN 15D

Succinic Acid, Gm./L.	Age of Culture, Days	Alkaloids in Medium, mg. %	Dry Weight of Mycelium, Gm.
2.7	35	2.94	...
2.7	40	2.46	...
2.7	45	1.57	4.32
5.4	35	5.40	...
5.4	40	3.67	...
5.4	45	4.53	4.32
10.8	35	11.48	...
10.8	40	20.25	...
10.8	45	36.72	5.95
21.6	35	3.99	...
21.6	40	7.21	...
21.6	45	12.69	5.74

of ascorbic acid in concentrations of 5, 10, and 20 mg./L. of Stoll-Abe medium was prepared. Two flasks of each vitamin concentration were inoculated with strain 15D, allowed to incubate thirty-five days, and the media assayed.

Taber and Vining (14) obtained evidence which led them to conclude that an exogenous source of biotin was necessary for growth of *C. purpurea*. It was decided, therefore, to test the influence of added biotin on alkaloid production by strain 15D. Two flasks of Stoll-Abe medium containing added biotin in a concentration of 100 μ g./L. were prepared, inoculated with strain 15D, allowed to incubate for thirty-five days, and assayed.

Enzymatic casein hydrolysate had been shown (8) to be a superior nitrogen source for growth and development of *C. purpurea* in culture. The effect of substituting it for the ammonium succinate in the Stoll-Abe formula was next tested. Two flasks of medium were prepared containing an equivalent weight of casein hydrolysate instead of the succinic acid and the pH was not adjusted by the addition of ammonium hydroxide. The flasks were inoculated with strain 15D, incubated, and the media assayed as previously described.

Claviceps purpurea is known to thrive in a large number of media, the composition of which is quite different from that of Stoll-Abe. To determine what effect the composition of some of these might have on alkaloid production, two were selected at random for testing. These were the casein hydrolysate-mannitol-basic nutrient medium of Tyler and Schwarting (8) and the carbohydrate-peptone medium of Gjerstad (15), modified by Adams (16) by the addition of 80 Gm. of mannitol, 20 mg. of indole acetic acid, and 10 additional Gm. of sucrose (20 Gm. total) per liter.

Two flasks of each of these media were inoculated with strain 15D and also two of the carbohydrate-peptone medium with strain 47. After incubating thirty-five days, the media of all cultures were assayed.

Averages of the results obtained in these various modifications and changes of the culture medium are reported in Table VI.

The Alkaloid Content of Mycelial and Pseudosclerotial Tissues.—Many cultures occasionally formed the dense black knots of tissue termed pseudosclerotia by McCrea (17); however, it was observed that such structures were particularly

TABLE VI.—THE INFLUENCE OF CERTAIN MEDIA MODIFICATIONS ON ALKALOID PRODUCTION BY CLAVINE-PRODUCING STRAINS

Medium	Volume of Medium, ml.	Alkaloids in Medium, mg. %	Dry Weight of Mycelium, Gm.
Ascorbic acid added			
5 mg./L.	93	2.89	...
10 mg./L.	90	2.62	...
20 mg./L.	90	2.30	...
Biotin added			
100 μg./L.	90	5.48	1.58
Casein hydrolysate substituted	88	2.03	1.62
Casein hydrolysate-mannitol-basic nutrient	90	1.11	0.84
Carbohydrate peptone			
strain 15D	89	22.14	4.19
strain 47	75	36.34	5.16

distinctive, characteristic, and abundant on the medium containing α-ketoglutaric acid substituted for succinic acid. Since it has been postulated that only true sclerotial tissue is capable of ergot alkaloid synthesis, at least under normal parasitic conditions (18), it seemed of interest to determine if there was any difference in alkaloid content between the pseudosclerotial and the mycelial tissues.

Eight flasks each of the Stoll-Abe medium and the medium in which α-ketoglutaric acid was substituted for the succinic acid were prepared, inoculated with strain 15D, and allowed to develop for thirty-five days. At the end of this time the tissue was removed from the flasks and carefully separated into mycelial and pseudosclerotial tissues produced on each medium. The pooled tissue types were dried, defatted, and subjected to duplicate analyses.

In the case of the tissues produced on the Stoll-Abe medium, no significant difference was found between the two types. The alkaloid content of the mycelium averaged 0.095%, that of the pseudosclerotia 0.091%. The alkaloid content of the pseudosclerotia produced on the α-ketoglutaric acid medium was more than three times as large (0.045%) as the content of the respective mycelium (0.013%).

Alkaloid Production in Shake Culture.—Preliminary experiments with the organism in shake culture yielded seemingly erratic results which were found to be due to a rapid increase, followed by an even more rapid decrease, of the alkaloid concentration of the medium under these conditions. Once this was understood, uniform results were obtained by using 2-L. Erlenmeyer flasks containing 500 ml. of the culture medium. The flasks were inoculated with strain 15D and placed on a New Brunswick Rotary Action Flask Shaker, Model V, operating at 280 r. p. m. Alkaloid formation was followed by withdrawing aseptically a few milliliters of medium at frequent intervals and assaying as previously described.

Since preliminary experiments had further indicated a rather low level of alkaloid production under

these conditions in the original Stoll-Abe medium, an experiment was undertaken to determine the effect of added mannitol on alkaloid production in submerged culture. Four flasks of Stoll-Abe medium containing 5, 10, 15, and 20% of mannitol respectively were prepared and tested with the results shown in Table VII.

DISCUSSION

Of a number of single carbohydrates tested as possible replacements for the mannitol in the Stoll-Abe medium, none produced higher alkaloid yields in the medium or the mycelium following a thirty-five-day fermentation period than the original sugar alcohol. When the medium was supplemented with 5% sucrose in addition to the mannitol, alkaloid production decreased substantially, thus indicating a possible difference in requirements for alkaloid biosynthesis between the *Agropyron* ergot strain employed, 15A, and the *Pennisetum* ergot strain of Stoll, *et al.*

Only one carbohydrate, sorbitol, proved approximately half as good as mannitol in terms of alkaloid concentration in the medium. Increasing the concentration of the mannitol did yield greater alkaloid concentrations, but growth and metabolism were considerably slower, and in the 20% mannitol medium maximum alkaloid production had apparently not been obtained after forty-five days.

Substitution of various organic acids likewise revealed none superior to succinic in terms of total alkaloid concentration of the medium. Several, including L-malic, citric, fumaric, glutamic, and α-ketoglutaric acid proved nearly as good on an equal weight basis, and of these, citric acid also yielded mycelium with an eightfold increase in alkaloids in comparison to that produced in the succinic acid medium.

These experiments do not, however, clearly differentiate between the importance of the anionic plant acid and its ammonium cation. Since nearly equivalent additions of ammonium hydroxide gave significantly different results in the presence of different acids, the latter are not without effect. Nevertheless, the exact roles of the cation and the anion remain to be evaluated.

Increasing the succinic acid concentration in combination with an increased mannitol concentration (15%) caused a further increase in alkaloid production, but growth and development of the organism were slowed considerably and again, at the higher concentrations, maximum alkaloid production was apparently not achieved after a forty-five-day fermentation period.

Additions of ascorbic acid to the Stoll-Abe medium depressed alkaloid production in proportion to the quantity added; biotin likewise apparently showed a slight negative activity, and the substitution of casein hydrolysate for succinic acid caused a decrease in alkaloid production under the conditions employed.

The casein hydrolysate-mannitol-basic nutrient medium proved decidedly inferior for alkaloid production, but the carbohydrate-peptone medium gave superior results to those obtained in the original Stoll-Abe medium for both strains 15D and 47. Yields obtained for strain 15D were still inferior to those achieved with increased mannitol and succinic acid concentrations in the Stoll-Abe medium.

The strongly differentiated, numerous pseudosclerotia produced on the medium containing α-ketoglutaric acid contained more than three times as much alkaloid as the accompanying mycelium. Nevertheless, their content was only about half as much as that of the mycelium from a standard succinic acid culture medium, in which the content of the less strongly differentiated pseudosclerotia did not differ appreciably from that of the mycelium.

Assuming that alkaloid production or concentration is actually greater in pseudosclerotial tissues, this apparent discrepancy might be explained by the fact that in the succinic acid cultures all of the growth more nearly approached the character of pseudosclerotial tissue than mycelial tissue. This was certainly indicated by a comparison of the appearance and form of the growth in the respective media.

Shaking speeded up the growth of the organism somewhat, but in the unmodified Stoll-Abe medium reduced the maximum alkaloid concentration of the culture liquid to about one-tenth that of the stationary cultures. The alkaloid content of the mycelial tissue produced in submerged culture was found to be negligible. Increasing the mannitol content of the shake medium also caused an appreciable increase in the alkaloid concentration of the nutrient liquid.

Apparently, submerged fermentation causes a speed-up in both the anabolism and catabolism of the clavine alkaloids, as evidenced by the rather limited period when these compounds accumulate to any appreciable extent. This most likely occurs when a stage of development of the organism is reached which greatly favors the anabolic phase of this dynamic equilibrium, but the increased catabolic activity is still present and the concentration of alkaloid never reaches a level equivalent to that achieved in stationary culture.

Regardless of the nutrient medium employed, the clavine alkaloid-producing cultures always synthesized detectable quantities of alkaloids. Chromatographic analysis revealed that the qualitative identity of the alkaloidal mixture did not vary with the different nutritional conditions, but if production was extremely small, it was sometimes difficult to detect those alkaloids which were present only as

TABLE VII.—THE EFFECT OF TIME AND THE MANNITOL CONCENTRATION ON ALKALOID PRODUCTION IN SHAKE CULTURE

Age of Culture, Days	Alkaloids in Medium, mg. %			
	5% Mannitol	10% Mannitol	15% Mannitol	20% Mannitol
10	0.41	1.03	0.54	0.11
12	0.62	0.75	0.59	0.44
14	0.70	1.11	0.75	0.48
17	1.56	4.59	3.81	3.24
19	1.19	5.62	5.02	4.70
21	1.13	5.83	5.62	6.35
24	1.30	6.48	9.02	9.07
26	1.57	6.70	9.40	10.04
28	1.08	7.08	11.02	12.05
31	0.65	2.16	3.85	4.32
33	Negligible	1.19	2.48	4.10

minor constituents of the total fraction. It is not unlikely that if production and testing had been carried out on an expanded scale, an even larger number of minor alkaloids might have been detected in the culture.

The quantitative production of alkaloids was shown to be influenced markedly by the concentration and identity of the carbohydrate source and the organic acid. It was also shown to fluctuate with time, ascending to a peak and then descending if the culture was allowed to continue metabolizing. The rapidity of this fluctuation was found to be dependent upon the composition of the medium, the cultural conditions (shake or stationary), and would no doubt vary also with different strains of *C. purpurea*.

A recent publication (6) has shown that it is possible to select strains of greatly increased alkaloid-producing capacity (150 mg. % in the nutrient medium). The productive ability of such strains can doubtlessly be increased still further by the selection of a proper nutrient medium based upon the fundamental observations and experimental data reported in this study.

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A Study of the Anthelmintic Activity of Some Lactones and Peroxides*

By DUANE G. WENZEL and CARROLL M. SMITH

Several lactones and peroxides were compared to santonin and ouabain for their *in vitro* anthelmintic activity. An unsuccessful attempt was made to correlate anthelmintic activity of the lactones and peroxide-forming ability. At a 1:1,000 concentration, tertiary-butyl hydroperoxide was the most active compound tested.

SEVERAL ANTHELMINTIC AGENTS including santonin contain an unsaturated lactone ring in their structure. While saturation of this ring has been found by some to decrease the effectiveness of santonin and simple lactones (1), the saturated compounds still retain some activity (1–3). Because unsaturation in a lactone may contribute to the formation of a peroxide (4) and because ascaridole, diheptanol peroxide, as well as the peroxides of olive, cottonseed, and peanut oil are active as anthelmintics (5), it was decided to examine the possibility of a relationship between the anthelmintic activity of simple lactones and their predictable peroxide formation. Several organic peroxides, santonin, and ouabain were also included in the study.

EXPERIMENTAL

Except for santonin, which was obtained from Merck and Company, the sources and structures of the compounds tested have been previously described (4).

The worms used were the parasitic intestinal nematode of the hog, *Ascaris suum*, collected shortly after the death of the hogs. They were washed in a special Ringer's solution (6) and maintained in this solution at 33–37°. This solution was also used as the bath medium in the studies described. Worms kept at the lower temperatures of the indicated range lived longer (from three to four days total) and generally produced more satisfactory recordings. Storage at temperatures of 37° and above weakened the worms and considerably hastened their death. Only female worms of a rose-pink color were used as these were found to be more vigorous and uniformly active.

Method.—Anterior and intermediate sections of the worms were prepared according to Baldwin (6). Test agents were added to the Ringer's solution bath maintained at a temperature of 37°. Movements of the worm sections were recorded kymographically. The anterior sections contain the "nerve-ring" in which are collected a majority of the ganglion cells of the nervous system. This apparently serves as a primitive brain and those drugs which are more active on the anterior section are believed to produce their effect on this nervous system. Santonin has been reported to be more effective on this preparation (6). In preliminary

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observations with the lactones it was found that better responses were also obtained with the anterior sections. For this reason all studies in this report were conducted using these sections.

Differences of opinion exist as to whether the worms should be stored and studied in oxygenated solutions or under relatively anaerobic conditions. While it has been stated that the ascarid lives in a nearly anaerobic environment and should be tested under similar circumstances (7), the statement has also been made that complete anaerobiasis is not obtained under the conditions of testing (8). Furthermore, the intestinal gases of the hog are reported to contain about 5% oxygen (9). Since the lactones are known to absorb oxygen in the formation of peroxides (10), initial tests were conducted in an oxygenated medium. It was observed that oxygenation *per se* was toxic to the worms and further tests were carried out in an ungassed bath. In order to determine the effect of an anaerobic environment on the actions of the compounds, $\Delta\beta$, γ-angelica lactone was also tested in a vacuum-degassed, nitrogen-treated bath.

Compounds were tested initially at a 1:1,000 concentration and the time required to produce paralysis recorded in minutes. If any appreciable activity was observed, they were re-examined at a 1:5,000 concentration. Santonin could not be studied at the higher concentration because of its limited solubility. The number of sections employed for each compound varied according to the uniformity of the results obtained.

RESULTS AND DISCUSSION

The effect of the test agents on the anterior preparations of the ascarid are listed in Table I. The values are recorded as the mean times to paralysis in minutes followed by the standard error for those experiments in which positive results were obtained with six or more preparations. Due to the varying sensitivity of the sections, some tests were equivocal and still others, using the same concentration of the compound, were essentially negative. These results are expressed respectively by a question mark and a minus sign. The most desirable and easily interpreted effect was the transition from active rhythmical contractions to a steady unmoving state. This was interpreted as paralysis. The onset of paralysis was preceded by a period of changing activity and it was not uncommon to observe short volleys of convulsive movements after normal activity had ceased. The character of this movement was such that it could not be termed purposeful and although it sometimes occurred in a somewhat rhythmic pattern, the worm section was still considered to be paralyzed. The test agent-induced transition from normal movement to paralysis and thence to the convulsive movements is illustrated by Fig. 1. A typical record illustrating the production of paralysis is shown by Fig. 2. Figures 3 and 4 are characteristic of responses termed equivocal. It is seen that definite changes in the amplitude or type of activity occur, but it could not be concluded that the action in these cases was that of an effective anthelmintic.

From an examination of the Table, it may appear that the β-methyl angelica lactones are more effective

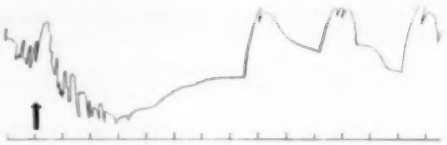

Fig. 1.—Paralyzant action of santonin 1:5,000. Typical volleys of convulsive movements are seen after the onset of paralysis. Time in 3.3-minute intervals.

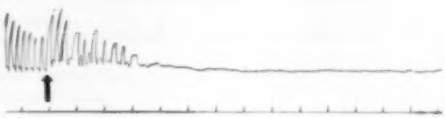

Fig. 2.—Paralyzant action of γ-ethyl $\Delta\alpha,\beta$angelica lactone, 1:1,000. Time in 3.3-minute intervals.

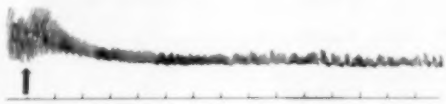

Fig. 3.—A typical equivocal anthelmintic response. $\Delta\alpha,\beta$angelica lactone, 1:1,000. Time in 5-minute intervals.

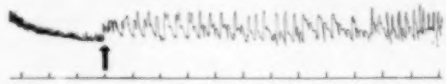

Fig. 4.—A typical equivocal anthelmintic response. γ-Ethyl-α,β angelica lactone, 1:5,000. Time in 5-minute intervals.

at a 1:5,000 concentration than at 1:1,000. It may be seen, however, that several of the sections at a 1:5,000 concentration produced equivocal responses. A similar situation exists for 2,2-bis (tertiary-butylperoxy) butane. Because only the most sensitive preparations responded at the lower concentrations, the percentage of sections yielding a positive response was always less at 1:5,000 than at the 1:1,000 concentration despite the fact that the times to paralysis apparently indicate an equal or greater activity.

The equivocal results obtained with the saturated lactones, γ-butyrolactone and γ-valerolactone, are consistent with the observation that the activity of santonin is reduced by two-thirds when the molecule is saturated (1).

Inasmuch as unsaturated lactones have been demonstrated to produce biological effects by reacting with amino or sulfhydryl groups of proteins (11), it cannot be concluded that the anthelmintic activity of the unsaturated lactones is necessarily related to peroxide formation. The similarity of the action of the peroxides tested to that of santonin and the simple unsaturated lactones may be related to the known ability of peroxides to oxidize sulfhydryl groups. Thus both types of compounds may act by reacting with sulfhydryl groups, the unsaturated lactones by addition across the double bond and the peroxides through the formation of

TABLE I.—ANTHELMINTIC ACTIVITY OF SOME LACTONES AND PEROXIDES

Compound[a]	Time in Minutes to Paralysis[b-e] 1:1,000	1:5,000
$\Delta^{\beta,\gamma}$Angelica lactone	25 (8) ± 6.1	57 (2)
	N/₂ 34 (7) ± 6.6	? (1)
	N_2 ? (1)	− (1)
$\Delta^{\alpha,\beta}$Angelica lactone	32 (6) ± 6.1	39 (2)
		? (2)
β-Methyl $\Delta^{\beta,\gamma}$angelica lactone	30 (7) ± 7.8	22 (1)
		? (3)
β-Methyl $\Delta^{\alpha,\beta}$angelica lactone	30 (9) ± 6.9	27 (3)
		? (1)
γ-Methyl $\Delta^{\alpha,\beta}$angelica lactone	34 (7) ± 7.3	? (3)
	? (1)	− (1)
γ-Ethyl $\Delta^{\alpha,\beta}$angelica lactone	28 (9) ± 5.4	? (6)
	? (1)	
γ-Butyrolactone	? (4)	
γ-Valerolactone	? (2)	
	− (1)	
Tertiary-butyl hydroperoxide	9 (7) ± 5.7	30 (9) ± 9.2
		? (7)
Di-tertiary-butyl peroxide	36 (3)	67 (1)
	? (3)	? (3)
2,2-Bis(tertiary-butylperoxy) butane	29 (7) ± 10.5	23 (2)
	? (2)	? (5)
Ouabain	63 (2)	
	? (2)	
	− (1)	
Santonin	Insoluble	30 (10) ± 7.4
		? (7)
		− (1)

[a] Structures are given in a previous publication (4).
[b] ± S. E. = $\sqrt{\Sigma d^2/N(N-1)}$.
[c] Number of preparations tested in parentheses.
[d] Equivocal results are expressed as ?.
[e] Negative results are expressed as −.
[f] Anaerobic conditions.

disulfide groups. In order to conclude that the activity of the lactones is related to peroxide formation, it should be necessary to correlate the anthelmintic activity with the theoretical capacity to form peroxides (4). This was not accomplished. It can only be stated that the more active peroxide-forming unsaturated lactones, i. e., those without γ-substitution in this case, were somewhat more effective as anthelmintics. This was most evident at the 1:5,000 concentration. Unfortunately, the same factors which influence peroxide-formation, particularly stabilization of free radical formation through resonance, also increase the reactivity of the compound toward thiol or amino groups.

Ouabain was found to be relatively inactive at a 1:1,000 concentration. Apparently the activity relationship observed between ouabain and the unsaturated lactones on the frog heart (4) does not apply to the anthelmintic action.

The effect of santonin at 1:5,000 was no greater than that exhibited by the unsaturated lactones either in terms of time to paralyze or per cent responding. A point of similarity observed with both agents was the frequent appearance of convulsive contractions after paralysis.

SUMMARY

1. A group of simple lactones, peroxides, santonin, and ouabain were tested *in vitro* for their paralytic effect on anterior sections of *Ascaris suum*.

2. All the unsaturated lactones and the peroxides demonstrated some anthelmintic activity. The activity of santonin at a 1:5,000 concentration was no greater than that of most of the unsaturated lactones. Ouabain was only slightly active. Tertiary butyl hydroperoxide was considerably more active than any other compound tested at a concentration of 1:1,000.

3. Because of the nature of the results and the problems inherent in evaluating them, it was not possible to conclude to what, if any, degree the activity of the lactones is related to peroxide formation.

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Synthesis and Biotesting of Metallic Surfactants*

By JACK N. BONE and DAVID W. O'DAY

The silver, mercury, lead, zinc, copper, and iron salts of lauryl sulfate, dioctyl sulfosuccinate, and isopropylnaphthalene sulfonate have been made. The activity of these has been determined against *Micrococcus pyogenes* var. *aureus*, *Pseudomonas aeruginosa*, and *Trichophyton mentagrophytes*. The activity of the copper, iron, zinc, and lead salts of the surfactants against the bacteria was increased over the activity of their corresponding chlorides, nitrates, or sulfates. There was a decrease in activity in the mercury and silver surfactants against the bacteria. The antifungal activity of all six metals was increased in the form of the surfactant salts as compared with their corresponding chloride, nitrate, or sulfate salts. Of the three surfactants used, the alkyl sulfate appeared to increase the activity of iron, mercury, and silver more than the other two against *Micrococcus aureus*. The alkyl sulfate salts of mercury and silver were more active against *Trichophyton mentagrophytes* than the same salts of the other two surfactants.

THE ACTIVITY of metals, such as mercury, silver, lead, copper, zinc, and iron, against bacteria and fungi is well known (1, 2). The antibacterial and antifungal properties of surface-active agents have been studied in great detail, especially the cationic surface-active agents. Since 1935 literally hundreds of these have been synthesized and their activity against bacteria and fungi examined. Although the cationic agents have received much more attention than the anionic surface-active agents, there are numerous antibacterials and antifungals found in the latter type (3–5). This knowledge led us to investigate the possibilities of preparing metallic salts of several anionic surface-active agents and of determining their antibacterial and antifungal properties. There is a rather extensive list of papers dealing with the preparation of and bacteriologic effects of anionic surface-active agents, but most of these pertain to the sodium, potassium, or ammonium salts. There is very little published work describing the preparation of other metallic or metallic-like salts of surfactants (6, 7) and very little, if any, describing their antibacterial and antifungal properties.

The purpose of this work was two fold; the preparation of metallic salts of various anionic surface-active agents, and the determination of their antibacterial and antifungal properties.

EXPERIMENTAL

Preparation of the Salts.—The metals selected for this investigation were silver, mercury, lead, zinc, copper, and iron. The surface-active agents selected to be used in the preparation of these metallic salts were sodium lauryl sulfate, U. S. P.;[1] sodium dioctyl sulfosuccinate;[2] and sodium isopropylnaphthalene sulfonate.[3] The above metallic salts of the alkyl sulfate were prepared by a modification of a method suggested by Lottermoser and Stoll (7). The U. S. P. grade sodium lauryl sulfate was dissolved in 95% ethanol and filtered to remove the excess sodium sulfate, the alcoholic solution then being evaporated to dryness. The sodium alkyl sulfate was dissolved in distilled water and barium chloride solution added to precipitate barium alkyl sulfate. This was removed by filtration, washed with distilled water, and dried. The metallic salts were prepared by refluxing together barium alkyl sulfate and the appropriate metallic sulfate in 95% ethanol for twenty-four hours. The mixture was filtered, and the filtrate evaporated to dryness. The metallic alkyl sulfates were recrystallized from 95% ethanol, washed with benzene, and recrystallized again from 95% ethanol.

The metallic salts of dioctyl sulfosuccinate and isopropylnaphthalene sulfonate were prepared by a method suggested by O'Donnell (8), in which the sodium salts of these surfactants, after being dissolved in 95% ethanol at approximately 5°, were treated with cold 40% sulfuric acid and the mixture allowed to stand in an ice bath for one hour. The precipitated sodium sulfate was filtered off and the filter cake was washed twice with small portions of cold alcohol. The proper metallic oxide, hydroxide, or carbonate was added and the mixture stirred while keeping cooled to 10°. Although the suggested reaction time was three hours, better yields were obtained by keeping the mixture at 10° from twenty-four to forty-eight hours. The excess oxide, hydroxide, or carbonate was filtered off and the filtrate evaporated to dryness.

Antibacterial Determination.—Two test organisms were used, the Gram-positive *Micrococcus pyogenes*, var. *aureus*, F. D. A. strain No. 209 (American Type Culture Collection No. 6538) and the Gram-negative *Pseudomonas aeruginosa*, A M S strain 25-A-2 (ATCC No. 8709). The

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[1] Duponol C, E. I. du Pont de Nemours and Co., Wilmington 98, Del.
[2] Aerosol OT, American Cyanamid Co., Industrial Chem. Division, Chicago 18, Ill.
[3] Aerosol OS, American Cyanamid Co., Industrial Chem. Division, Chicago 18, Ill.

Reddish method (9) of testing antiseptics was used, except that sodium chloride was omitted from the nutrient broth. When the test culture grown in the nutrient broth containing sodium chloride was added to a solution of the silver salts of the surfactants, a white precipitate was formed. In order to use the test organisms grown in similar media for all tests, the sodium chloride was omitted from the nutrient broth. Reactions of the organisms to various aqueous dilutions of the metallic surfactants were tested at 37°. The result shown in Tables I, II, and III are averages of four or more determinations and give the maximum dilution killing in five minutes or less, the maximum dilution not killing in five minutes but killing in ten minutes, and the strongest concentration which would not kill in fifteen minutes. In some instances, these could not be determined. As a comparison, the sulfate, chloride, or nitrate salts of these metals were tested against *M. aureus* and *Ps. aeruginosa*. The results are shown in Table IV.

Antifungal Determination.—The test organism used was *Trichophyton mentagrophytes*, ATCC No. 9533, strain No. 640. A slight modification of the Burlingame and Reddish (10) test method was used. The test organism to be used was grown on Sabouraud's agar for five days at 28° ± 2°. The organism was then streaked over the entire surface of Sabouraud's agar (20 cc.) in a 9-cm. Petri dish by means of a sterile cotton swab inoculated with the test culture. These plates were incubated at 28° ± 2° for five days, then cut into disks 1 cm. in diameter by means of a sterile cork borer. One disk was removed as a control, then the surface of the culture was flooded with 20 cc. of the solution or suspension (25°) to be tested. At the end of each five, ten, or fifteen-minute test period, one of the disks was removed and placed in 10 cc. of sterile, distilled water. The excess test solution was washed from the disk by gentle agitation during a two-minute period. The culture disk was removed from the wash water and placed, with the culture

TABLE I.—ANTIBACTERIAL ACTIVITY OF METALLIC SALTS OF ALKYL SULFATE

	M. aureus				*Ps. aeruginosa*			
		Minutes				Minutes		
Salt	Dilution	5	10	15	Dilution	5	10	15
Copper	1:1,000	–[a]	–	–	1:200	–	–	–
	1:2,000	+	–	–	1:300	+	–	–
	1:4,000	+	+	+	1:400	+	+	+
Iron	1:3,000	–	–	–	1:200	–	–	–
	1:4,000	+	–	–	1:300	+	–	–
	1:5,000	+	+	+	1:400	+	+	+
Mercury	1:10,000	–	–	–	1:10,000	–	–	–
	1:16,000	+	–	–	1:14,000	+	–	–
	1:20,000	+	+	+	1:16,000	+	+	+
Silver	1:4,000	–	–	–	1:1,000	–	–	–
	1:6,000	+	–	–	1:2,000	+	–	–
	1:8,000	+	+	+	1:4,000	+	+	+
Zinc	1:200	–	–	–	1:200	–	–	–
	1:300	+	–	–	1:300	+	–	–
	1:400	+	+	+	1:500	+	+	+
Lead	1:200	–	–	–	1:200	–	–	–
	1:300	+	–	–	1:300	+	–	–
	1:400	+	+	+	1:400	+	+	+
Sodium	1:20	–	–	–	1:20	+	+	+
	1:50	+	–	–				
	1:100	+	+	+				

[a] – No growth. + Growth.

TABLE II.—ANTIBACTERIAL ACTIVITY OF METALLIC SALTS OF DIOCTYL SULFOSUCCINATE

	M. aureus				*Ps. aeruginosa*			
		Minutes				Minutes		
Salt	Dilution	5	10	15	Dilution	5	10	15
Copper	1:1,300	–[a]	–	–	1:300	–	–	–
	1:1,400	+	–	–	1:500	+	–	–
	1:1,500	+	+	+	1:800	+	+	+
Iron	1:900	–	–	–	1:400	–	–	–
	1:1,000	+	–	–	1:500	+	–	–
	1:1,200	+	+	+	1:600	+	+	+
Mercury	1:1,200	–	–	–	1:1,500	–	–	–
	1:1,400	+	–	–	1:1,600	+	–	–
	1:1,600	+	+	+	1:1,800	+	+	+
Silver	1:3,000	–	–	–	1:100	–	–	–
	1:4,000	+	–	–	1:200	+	–	–
	1:5,000	+	+	+	1:300	+	+	+
Zinc	1:100	+	+	+	1:100	+	+	+
Lead		[b]	[b]	[b]				
Sodium	1:20	+	+	+	1:20	+	+	+

[a] – No growth. + Growth. [b] Insoluble in water.

TABLE III.—ANTIBACTERIAL ACTIVITY OF METALLIC SALTS OF ISOPROPYLNAPHTHALENE SULFONATE

	M. aureus				*Ps. aeruginosa*			
		Minutes				Minutes		
Salt	Dilution	5	10	15	Dilution	5	10	15
Copper	1:800	−[a]	−	−	1:200	−	−	−
	1:1,000	+	−	−	1:300	+	−	−
	1:1,200	+	+	+	1:400	+	+	+
Iron	1:700	−	−	−	1:100	+	+	+
	1:800	+	−	−				
	1:1,000	+	+	+				
Mercury		[b]	[b]	[b]				
Silver	1:3,000	−	−	−	1:2,000	−	−	−
	1:4,000	+	−	−	1:3,000	+	−	−
	1:5,000	+	+	+	1:4,000	+	+	+
Zinc	1:200	−	−	−	1:100	+	+	+
	1:300	+	−	−				
	1:500	+	+	+				
Lead		[b]	[b]	[b]				
Sodium	1:20	+	+	+	1:20	+	+	+

[a] − No growth. + Growth. [b] Insoluble in water.

TABLE IV.—ANTIBACTERIAL ACTIVITY OF SULFATE, CHLORIDE, OR NITRATE METALLIC SALTS

	M. aureus				*Ps. aeruginosa*			
		Minutes				Minutes		
Salt	Dilution	5	10	15	Dilution	5	10	15
Copper sulfate	1:50	+[a]	+	+	1:50	+	+	−
					1:100	+	+	−
					1:200	+	+	+
Ferrous sulfate	1:50	+	+	+	1:50	+	+	+
Ferric sulfate	1:50	+	+	+	1:100	−	−	−
					1:200	+	−	−
					1:400	+	+	+
Mercuric chloride	1:16,000	−	−	−	1:16,000	−	−	−
	1:32,000	+	−	−	1:25,000	+	−	−
	1:64,000	+	+	+	1:64,000	+	+	+
Silver nitrate	1:8,000	−	−	−	1:8,000	−	−	−
	1:12,000	+	−	−	1:14,000	+	−	−
	1:20,000	+	+	+	1:30,000	+	+	+
Zinc sulfate	1:50	+	+	+	1:50	+	+	+
Lead chloride	1:50	+	+	+	1:50	+	+	+

[a] − No growth. + Growth.

side down, on the surface of Sabouraud's agar in a Petri dish. These dishes were inverted, incubated at 28° ± 2°, and observed for growth for a maximum of three weeks. The results shown in Table V are averages of four or more determinations, and give the maximum dilution killing in five minutes or less, the maximum dilution not killing in five minutes but killing in ten minutes, and the strongest concentration which would not kill in fifteen minutes. In some instances, these could not be determined. As a comparison, the sulfate, chloride, or nitrate salts of these metals were tested against *Tr. mentagrophytes*. The results are shown in Table VI.

DISCUSSION

Reddish (1) states that "the information desired is the concentration of the antiseptic required to kill *M. pyogenes* var. *aureus* under the conditions of the test within five minutes. If a preparation does not pass this test within this time period, it is not sufficiently germicidal to be classified as an antiseptic. Those preparations that do pass the test within five minutes or less, must then be submitted to further study under practical and clinical conditions."

Using the above criterion in this "screening test," the results show that copper sulfate in a 2% solution does not exhibit antibacterial activity but does show such activity in the form of copper dioctyl sulfosuccinate in a 1:1,300 solution against *M. aureus* and 1:300 solution against *Ps. aeruginosa*. Ferrous sulfate and ferric sulfate in a 2% solution did not kill *M. aureus*, but the iron alkyl sulfate was effective in a 1:3,000 solution. Two per cent ferrous sulfate solution was inactive against *Ps. aeruginosa*, while a 1% ferric sulfate showed activity. The iron salt of dioctyl sulfosuccinate was effective in a 1:400 concentration. A 2% solution of zinc sulfate did not kill either organism, but in the form of alkyl sulfate did kill both organisms in a 1:200 concentration. The same results as with the zinc were obtained in comparing lead chloride and lead alkyl sulfate. The lead salts of dioctyl sulfosuccinate and isopropylnaphthalene sulfonate were water insoluble and of such form that uniform suspensions could not be produced in distilled water. Mercury in the chloride salt and silver in the nitrate salt were more effective than in the surfactant salts against both organisms. Mercury isopropylnaphthalene sulfonate was water insoluble and of such form that a

TABLE V.—ACTIVITY OF METALLIC SURFACTANTS AGAINST *Trichophyton mentagrophytes*

	Alkyl Sulfate				*Dioctyl Sulfosuccinate*				*Isopropylnaphthalene Sulfonate*			
		Minutes				Minutes				Minutes		
Salt	Dilution	5	10	15	Dilution	5	10	15	Dilution	5	10	15
Copper	1:20	–[a]	–	–	1:20	–	–	–	1:20	–	–	–
	1:50	+	–	–	1:50	+	–	–	1:50	+	–	–
	1:100	+	+	+	1:100	+	+	+	1:100	+	+	+
Iron	1:20	–	–	–	1:20	–	–	–	1:20	+	+	+
	1:30	+	–	–	1:40	+	–	–				
	1:100	+	+	+	1:100	+	+	+				
Mercury	1:100	–	–	–	1:20	–	–	–	[b]			
	1:200	+	–	–	1:30	+	–	–				
	1:300	+	+	+	1:100	+	+	+				
Silver	1:100	–	–	–	1:20	–	–	–	1:20	–	–	–
	1:150	+	–	–	1:75	+	–	–	1:50	+	–	–
	1:200	+	+	+	1:150	+	+	+	1:100	+	+	+
Zinc	1:20	+	+	+	1:20	–	–	–	1:20	–	–	–
					1:40	+	–	–	1:30	+	–	–
					1:100	+	+	+	1:100	+	+	+
Lead	1:20	–	–	–	1:20	–	–	–	[b]			
	1:50	+	–	–	1:50	+	–	–				
	1:100	+	+	+	1:100	+	+	+				
Sodium	1:20	+	+	+	1:20	+	+	+	1:20	+	+	+

[a] – No growth. + Growth. [b] Insoluble in water.

TABLE VI.—ACTIVITY OF METALLIC CHLORIDE, NITRATE, OR SULFATE SALTS AGAINST *Trichophyton mentagrophytes*

		Minutes		
Salt	Dilution	5	10	15
Copper sulfate	1:20	+[a]	+	+
Ferrous sulfate	1:20	+	+	+
Ferric sulfate	1:20	+	+	+
Mercuric chloride	1:100	+	–	–
	1:250	+	+	+
Silver nitrate	1:50	+	–	–
	1:100	+	+	+
Zinc sulfate	1:20	+	+	+
Lead chloride	1:100	+	+	+

[a] – No growth. + Growth.

uniform aqueous suspension could not be made. Five per cent sodium alkyl sulfate solution exhibited activity against *M. aureus*, but not against *Ps. aeruginosa*. Five per cent sodium dioctyl sulfosuccinate and 5% sodium isopropylnaphthalene sulfonate solution were ineffective against both organisms.

In comparing the effectiveness of each of the three surfactants to potentiate the antibacterial activity of the metals, a study of the results showed very little difference between the three, with the exception of iron alkyl sulfate, which had considerably more activity against *M. aureus* than the other two iron surfactant salts. Although the alkyl sulfate did not potentiate mercury and silver activity, the alkyl sulfates of these two metals were more active than the other mercury and silver surfactant salts.

The antifungal test used was one of the most rigid *in vitro* tests employed in that a five-day-old culture was used. The growing fungus is killed rather than having its growth retarded as is done in some of the other tests. Exposure to 5% aqueous solutions of copper sulfate, ferrous sulfate, ferric sulfate, and zinc sulfate for five minutes at 25° did not kill *Trichophyton mentagrophytes*. One per cent mercuric chloride, 2% silver nitrate, and 1% lead chloride did not kill the fungus either. Five per cent aqueous solutions or suspensions of the copper, iron, zinc, and lead salts of one or more of the surfactants did kill the fungus after five minutes of exposure at 25°. One per cent aqueous solutions of mercury and silver alkyl sulfate also killed the organisms.

No appreciable difference was noted between the three surfactants to potentiate the antifungal activity of the metals with the exception of alkyl sulfate salts of mercury and silver. These appeared to have more activity than the corresponding salts of the other two surfactants.

SUMMARY

1. Silver, mercury, lead, zinc, copper, and iron salts of lauryl sulfate, dioctyl sulfosuccinate, and isopropylnaphthalene sulfonate have been made.

2. When tested *in vitro* against *M. pyogenes* var. *aureus* and *Ps. aeruginosa*, the copper, iron, zinc, and lead salts of these surfactants were more active than the corresponding chloride, nitrate, or sulfate salts. There was a decrease in activity in the mercury and silver surfactants against the bacteria as compared with mercuric chloride or silver nitrate.

3. The antifungal activity of all six metals in the form of the surfactant salts, when tested *in vitro* against *Trichophyton mentagrophytes*, was increased over the activity of their corresponding chloride, nitrate, or sulfate salts.

4. No appreciable difference was noted between the three surfactants to potentiate the antibacterial or antifungal activity of the metals with the exception of the iron alkyl sulfate being more active against *M. pyogenes* than the same iron salts of the other two surfactants. It was observed also that, although the alkyl sulfate did not potentiate mercury and silver over mercuric chloride or silver nitrate, the alkyl sulfate salts of

these two metals were more active against *M. pyogenes* than the same metallic salts of the other two surfactants. The alkyl sulfate salts of mercury and silver were also more active against the fungus than the same metallic salts of the other two surfactants.

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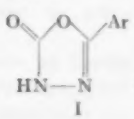
The Synthesis and Proof of Structure of N-Alkylated Oxadiazolones*

By HENRY C. CALDWELL†, ROBERT J. SEIWALD‡, and J. H. BURCKHALTER

The synthesis of seventeen 4-dialkylaminoalkyl-2-aryl-Δ^2-1,3,4-oxadiazolin-5-ones from 2-aryl-Δ^2-1,3,4-oxadiazolin-5-ones is presented. Assignment of the structures of these compounds is discussed.

DURING the past decade, many aryl hydrazides have been prepared for evaluation as tuberculostats. It was recently demonstrated that the oxadiazolone derived from isonicotinic acid hydrazide (INH) and phosgene was more effective as an antituberculous agent *in vivo* than INH (1, 2). Further, evidence has been presented to suggest that iproniazid, 1-isonicotinyl-2-isopropylhydrazine phosphate (Marsilid phosphate), is an effective hypotensive agent (3), and it is also recommended as a psychic energizer. In view of the interesting activities exhibited by hydrazides and the closely related oxadiazolones, the preparation of disubstituted oxadiazolones (II) for evaluation as pharmacodynamic and chemotherapeutic agents became of interest.



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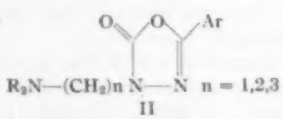

II

The parent oxadiazolones (I) were readily obtained by treating the appropriate aryl hydrazide with an excess of phosgene (2). The resulting product, 2-aryl-Δ^2-1,3,4-oxadiazolin-5-one (I), is in tautomeric equilibrium with 2-aryl-5-hydroxy-1,3,4-oxadiazole. It is evident, therefore, that the anion (III) derived from this compound could yield a mixture of two products (IV and V) when subjected to electrophilic attack by alkyl halides. Anions similar to III, which possesses two different reactive positions, have been named ambient anions by Kornblum (4). In general, silver salts strongly favor O-alkylation whereas alkali metal salts give N-alkylated products.

Silver oxide, potassium carbonate, and sodamide were allowed to react with the oxadiazolone I (Ar = C_6H_5). When the salts were treated separately with β-dimethylaminoethyl chloride, only one product was isolated and the same material was obtained in all cases. Table I summarizes the result.

Table I indicates that the N-alkylated compound IV was obtained. An infrared absorption spectrum verified these results, since a strong carbonyl peak was observed at 1770 cm.$^{-1}$. These findings are consistent with those of Kornblum. Further support for N-alkylation was afforded by a 66 per cent yield of II (Ar = phenyl, n = 3) from γ-dimethylaminopropyl chloride, sodamide, and the oxadiazolone.

TABLE I

Metallic Base	% Yield
Silver oxide	6
Potassium carbonate	50
Sodamide	76

* Received April 25, 1958, from the Department of Pharmaceutical Chemistry, University of Kansas, School of Pharmacy, Lawrence.

Extracted from a portion of the Ph.D. Thesis of Henry C. Caldwell, 1957.

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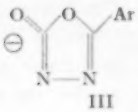
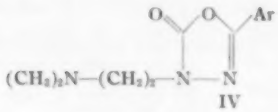
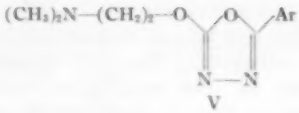


III



IV



V

A series of 15 Mannich bases (Table II) were prepared by treating the appropriate oxadiazolone with formalin and various secondary amines. When the secondary amine was piperazine, the product had the general structure VIb.[1]

When the aryl group is *p*-hydroxyphenyl, the Mannich side chain might be expected to enter either the carbocyclic or the heterocyclic ring giving either VIII or IX. Conceivably, both VIII and IX could be prepared by the Mannich reaction using VII under different experimental conditions. The Mannich base of phthalimide, which is similar to IX, was prepared by Hellmann and Loschmann (6) by allowing the reactants to stand for one hour at room temperature. Under the same conditions, a substance of m. p. 209–210° was obtained and presumed to be IX. Mannich bases from a number of phenols were prepared by Burckhalter, *et al.* (7), by dissolving the reactants in alcohol and heating at refluxing temperatures on the steam bath for several hours. When these conditions were employed, a product of m. p. 212–213° was obtained and tentatively assigned the structure VIII. A mixed melting point of the two products showed no depression. It is evident, therefore, that the same substance was formed in both reactions.

Since it has been demonstrated that methylene diamines, which are similar to VI, are susceptible to acid hydrolysis (8), one would expect that IX might be converted to the starting materials by the action of dilute hydrochloric acid. Hence, a sample of the solid of m. p. 212–213° was suspended in distilled water, and dilute aqueous hydrochloric acid was added with shaking until the solution was acidic to litmus. Most of the solid went into solution. When the solution was heated, a copious precipitate was obtained, m. p. 272–273°. A mixed melting point with the parent oxadiazolone VII showed no depression. This suggests that IX and not VIII was the product of both reactions, for the carbon to carbon bond of IX would presumably be stable under these conditions.

Since the heterocyclic ring was preferentially attacked, it was of interest to treat one equivalent of oxadiazolone VII with two equivalents of formalin and morpholine to determine whether the disubstituted Mannich base X could be obtained. Following the method of Burckhalter, *et al.* (7), the reactants were heated at reflux temperature for five hours. There was obtained a white product, m. p. 209–210°. A mixed melting point with the monosubstituted Mannich base IX showed no depression. Thus, the aromatic ring did not undergo substitution under forcing conditions.

It has been demonstrated by other workers (8–11) that Mannich bases of this general type will rearrange or react with certain other substances to form more stable products. The possible rearrangement, then, of IX to VIII was of interest. Mannich base IX was dissolved in alcohol and the solution was heated at reflux temperature for twelve hours. All the starting material was recovered. When the foregoing experiment was repeated with the addition of two drops of dilute hydrochloric acid, starting material IX was recovered.

During the course of the preparation of the Mannich bases (VIa), it was desirable to synthesize the analogous hydroxymethyl compound (XI). Heating I (Ar = phenyl) with an equivalent amount of formalin in dilute acetic acid gave XI in 70% yield. Repeated recrystallization from alcohol resulted in decomposition as noted by a gradual lowering of the melting point.

Pharmacological Results.—4-(β-Dimethylaminoethyl)-2-phenyl-Δ^2-1,3,4-oxadiazolin-5-one hydrochloride (II) demonstrated hypotensive activity in the perinephritic rat when administered orally but it was insufficient for further interest. The same compound, as well as its γ-dimethylaminopropyl homolog, was inactive *in vitro* against *M. tuberculosis*.

Compound 7 of Table II exhibited *in vitro* activity against *M. tuberculosis* but it was only about $^1/_{20}$ that of isoniazid, while compounds 6 and 8 were inactive.

EXPERIMENTAL[2]

2-Phenyl-Δ^2-1,3,4-oxadiazolin-5-one.—Wilder Smith prepared this substance, but he gave no experimental details (2). A solution containing 5 Gm. (0.036 mole) of benzoylhydrazide (13) in 80 ml. of dioxane was slowly dropped into 50 ml. of dioxane

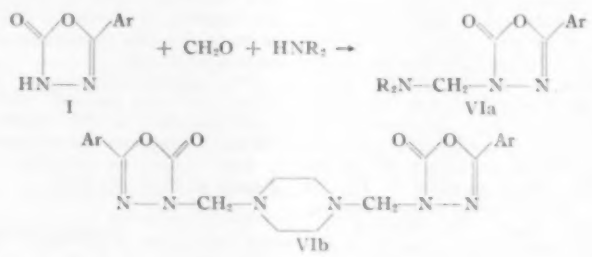
[1] After the completion of our studies, it was found that Dornow and Lüpfert had prepared three compounds of the same type for tuberculostatic tests (5).

[2] Melting points are uncorrected.

TABLE II.—4-DIALKYLAMINOMETHYL-2-ARYL-Δ^3-1,3,4-OXADIAZOLIN-5-ONES

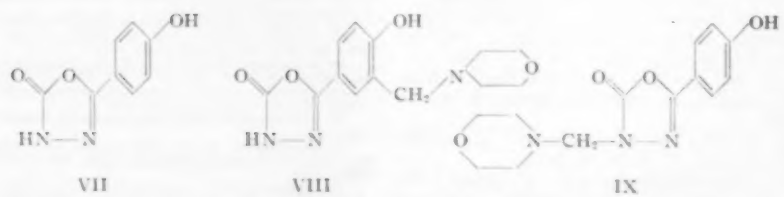
No.	Amino Group	Aryl Group	M. P.,[a] °C.	Yield, %	Formula	Analyses C Calcd.	C Found	H Calcd.	H Found
1	4-Morpholinyl	Phenyl	129–130	70	$C_{13}H_{15}N_3O_3$	59.76	59.83	5.79	5.83
2	1-Methylpiperazinyl	Phenyl	112–113	75	$C_{14}H_{18}N_4O_2$	61.30	61.42	6.61	6.60
3	Dimethylamino	Phenyl	97–98[b]	78	$C_{11}H_{13}N_3O_2$	60.26	60.41	5.98	6.03
4	Piperazinyl[c]	Phenyl	257–258	97	$C_{26}H_{32}N_6O_4$	60.82	60.92	5.11	5.26
5	4-Morpholinyl	4-Pyridyl	170.5–172	63	$C_{12}H_{14}N_4O_3$	54.95	54.90	5.38	5.48
6	1-Methylpiperazinyl	4-Pyridyl	144–144.5	97	$C_{13}H_{17}N_5O_2$	56.71	56.76	6.23	6.36
7	Piperazinyl[c]	4-Pyridyl	243–243.5	89	$C_{24}H_{30}N_8O_4$[e]	54.48	54.49	4.66	4.91
8	4-Morpholinyl	2-Phenyl-4-quinolyl	210.5–213	93	$C_{22}H_{20}N_4O_3$	68.03	67.94	5.19	5.17
9	1-Pyrrolidyl	2-Phenyl-4-quinolyl	159–160.5	92	$C_{22}H_{20}N_4O_2$	70.95	70.28	5.41	5.38
10	1-Methylpiperazinyl	2-Phenyl-4-quinolyl	162–163	95	$C_{23}H_{23}N_5O_2$	68.81	68.48	5.77	5.34
11	Piperazinyl[c]	2-Phenyl-4-quinolyl	252–253	93	$C_{42}H_{42}N_8O_4$	69.75	69.97	4.68	5.09
12	4-Morpholinyl	4-Hydroxyphenyl[d]	209–210	97	$C_{13}H_{15}N_3O_4$	56.31	56.63	5.45	5.22
13	1-Methylpiperazinyl	4-Hydroxyphenyl	179–180	94	$C_{14}H_{18}N_4O_3$	57.92	57.90	6.25	6.31
14	1-Pyrrolidinyl	4-Hydroxyphenyl	173–174	98	$C_{13}H_{15}N_3O_3$	59.76	59.36	5.79	5.70
15	Piperazinyl[c]	4-Hydroxyphenyl	251–252	96	$C_{26}H_{32}N_6O_6$	56.64	55.75	4.76	5.55

[a] Melting points are uncorrected. [b] Ref. 5 gives 93°. [c] For structure, refer to general structure VIb. [d] For intermediate oxadiazolone, see ref. 12. [e] Analysis corresponds to $^1/_4H_2O$.

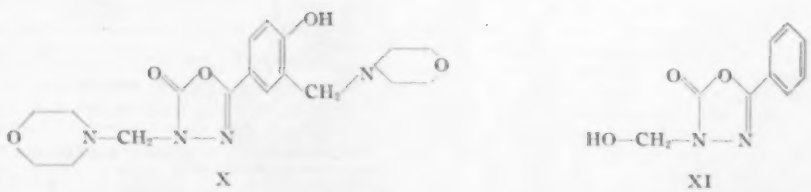


I VIa

VIb



VII VIII IX



X XI

which had previously been saturated with phosgene. Phosgene (HOOD) was continuously bubbled into the reaction mixture. The addition of the hydrazide required thirty minutes. Phosgene was bubbled into the solution for an additional fifteen minutes. The solvent and excess phosgene were then removed under reduced pressure. The product was recrystallized either from hot water or benzene-Skelly B. After drying, 5 Gm. (85% yield) of white solid, m. p. 137–138°, was obtained. Wilder Smith reported m. p. 138°.

2-(4-Pyridyl)-Δ^3-1,3,4-oxadiazolin-5-one.—Wilder Smith prepared this substance but gave no experimental details (2). A solution of 9.6 Gm. (0.07 mole) of isonicotinic acid hydrazide in 90 ml. of 3% aqueous hydrochloric acid was cooled in an ice bath and a stream of phosgene was passed through for thirty minutes. A copious precipitate of amine hydrochloride was isolated by filtration and the process was repeated. The salt was dissolved in a minimum amount of water, neutralized with potassium carbonate, and filtered. There

was obtained 10.5 Gm. (92% yield) of a cream-colored product, m. p. 270–272° (lit. 265°).

2-(2-Phenyl-4-quinolinyl)-Δ^2-1,3,4-oxadiazolin-5-one.—A warm mixture of 10 Gm. (0.038 mole) of 2-phenylcinchoninic acid hydrazide (14) and 200 ml. of dioxane was slowly added to 50 ml. of dioxane which had previously been saturated with phosgene. A heat lamp was placed near the dropping funnel to prevent crystallization of the hydrazide. The addition required thirty minutes and phosgene was passed into the reaction mixture for fifteen minutes longer. The solvent and excess phosgene were then removed under reduced pressure. The product was recrystallized from 80% alcohol. There was obtained 9.1 Gm. (83% yield) of a white solid, m. p. 233.5–234.5°. Another recrystallization from dilute alcohol gave the analytical sample, m. p. 234–234.5°.

Anal.—Calcd. for $C_{17}H_{11}N_3O_2$: C, 70.58; H, 3.83. Found: C, 70.40; H, 3.96.

4-(β-Dimethylaminoethyl)-2-phenyl-Δ^2-1,3,4-oxadiazolin-5-one Hydrochloride.—(A) To a solution of 1.6 Gm. (0.011 mole) of β-dimethylaminoethyl chloride hydrochloride (15) in a minimum amount of water, concentrated sodium hydroxide was added. The mixture was extracted with three different portions of benzene, and the combined extract dried over anhydrous potassium carbonate (15). (B) A mixture of 1.62 Gm. (0.01 mole) of 2-phenyl-Δ^2-1,3,4-oxadiazolin-5-one, 0.41 Gm. (0.011 mole) of sodamide, and 70 ml. of dry benzene was stirred and heated at refluxing temperature for two hours for the formation of the sodium salt of the oxadiazolone. The solution prepared in Part A was added slowly. Stirring and refluxing were continued for nineteen hours. The mixture was cooled and the solid sodium chloride was separated by filtration. The hydrochloride was prepared by treating the benzene solution with excess hydrogen chloride. After cooling overnight, there was obtained 2.4 Gm. (76% yield) of a white solid melting at 185–190°. After three recrystallizations from absolute alcohol, an analytical sample melted at 209–210°.

Anal.—Calcd. for $C_{13}H_{18}N_3O_2$.HCl: C, 53.43; H, 5.98. Found: C, 53.09; H, 6.11.

4-(γ-Dimethylaminopropyl)-2-phenyl-Δ^2-1,3,4-oxadiazolin-5-one Hydrochloride.—Adhering to the foregoing procedure, 3.24 Gm. (0.02 mole) of 2-phenyl-Δ^2-1,3,4-oxadiazolin-5-one, 3.8 Gm. (0.024 mole) of γ-dimethylaminopropyl chloride hydrochloride and 0.82 Gm. (0.022 mole) of sodamide gave 3.9 Gm. (66% yield) of white product, m. p. 163–164°. After two recrystallizations from acetone-Skelly B, an analytical sample melted at 167–169.5°.

Anal.—Calcd. for $C_{13}H_{17}N_3O_2$.HCl.$\frac{1}{2}H_2O$: C, 53.33; H, 6.54. Found: C, 53.31; H, 6.56.

4-Dialkylaminomethyl-2-aryl-Δ^2-1,3,4-oxadiazolin-5-ones (Table II).—These compounds were prepared by the general procedure of suspending the oxadiazolone in absolute alcohol and adding equivalent amounts of the amine and formalin with stirring. The reaction mixture became warm and usually complete solution was effected. The mixture was allowed to sit at room temperature for one hour. Most of the products were recrystallized from alcohol. However, in certain cases where they were insoluble in the common solvents, analytical samples were prepared by suspending the products in boiling alcohol in an effort to dissolve the impurities. The suspension was filtered and the solid was dried at room temperature.

4-Hydroxymethyl-2-phenyl-Δ^2-1,3,4-oxadiazolin-5-one (XI).—The following procedure is similar to the method employed by Burckhalter, Stephens, and Hall (16) for the preparation of the hydroxymethyl derivative of benzotriazole. A mixture of 1.62 Gm. (0.01 mole) of 2-phenyl-Δ^2-1,3,4-oxadiazolin-5-one, 0.8 ml. (0.01 mole) of 40% formalin, 3 ml. of glacial acetic acid, and 4 ml. of water was heated on the steam bath for thirty minutes. The product, which precipitated from the hot solution, was dried to give 1.35 Gm. (70% yield) of white solid melting at 159–161°. An analytical sample, prepared by two recrystallizations from alcohol, melted at 157–159°.

The reaction was repeated as above, but the acetic acid was omitted. The product was recrystallized from alcohol. A mixed melting point of the two products showed no depression.

Anal.—Calcd. for $C_9H_8N_2O_3$: C, 56.25; H, 4.19. Found: C, 56.14; H, 4.31.

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The Effects of Atropine and 2,4-Dichlorophenoxyacetic Acid on Leaf Alkaloid Accumulation in Certain Members of the *Solanaceae**

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Toxic concentrations of atropine and 2,4-dichlorophenoxyacetic acid were administered to *Datura stramonium* and *Lycopersicon esculentum*. The atropine in concentrations of 0.25 and 0.1 per cent was administered by addition to the culture media and the 2,4-D (0.01 per cent solution) was sprayed on the leaves. Characteristic growth responses were induced in both plants. Significant increases in the leaf accumulation of alkaloids were also induced in both species by the atropine treatments, and the effects of 2,4-D on this alkaloid accumulation are described.

THE ADDITION of large amounts of atropine sulfate to culture media of *Datura stramonium* has been shown to retard growth (1, 2). On the other hand, smaller quantities (less than 0.01 per cent) had no effect on growth or alkaloid formation (2). The influence of 2,4-D and other compounds on the growth and alkaloid formation in certain members of the *Solanaceae* family have similarly been observed (2–7). In general, varying results have been reported regarding alkaloid accumulation when plants have been treated with 2,4-D.

The purpose of this investigation was to determine the accumulation of atropine by *Datura stramonium* and *Lycopersicon esculentum* when large amounts of this alkaloid are added to culture substrates and plants are under the effects of 2,4-D. It would seem that the effects on accumulated alkaloid under these conditions might reveal more clearly a change if the alkaloid chemistry was affected by the growth regulator.

EXPERIMENTAL

Arrangement During Growth and Treatment.—Healthy Stramonium plants (*Datura stramonium* L.) and tomato plants (*Lycoperiscon esculentum* var. Bonnie Best, originator—George W. Middleton) were grown from seed in soil under greenhouse conditions. Upon reaching approximately 15 cm. in height, these were carefully transplanted to nutrient culture media in 1.5-L. wide mouth bottles. The culture medium consisted of a modified formula of Hoagland (8). Chemically pure reagents consisting of the following formula were employed in preparing the culture media: 0.005 *M* $Ca(NO_3)_2$, 0.005 *M* KNO_3, 0.002 *M* $MgSO_4$, 0.001 *M* KH_2PO_4, 0.00002 *M* ferric tartrate–$Fe_2(C_4H_4O_6)_3 \cdot H_2O$, 0.00005 *M* H_3BO_3, 0.00001 *M* $MnCl_2 \cdot 4H_2O$, 0.000001 *M* $ZnCl_2$, and 0.0000003 *M* $CuCl_2 \cdot 2H_2O$. The culture media were continuously aerated from an airline during the experiments. When transplanted to the culture media the *Datura* seedlings were about forty days old, calculated from the date of germination, while the tomato seedlings were about twenty-three days old. The pH of the freshly prepared culture solution was 5.2. During the growth period the old culture solution, the pH of which from random sampling averaged less than 7.0, was completely replaced at weekly intervals with a fresh Hoagland's solution. Excellent growth resulted.

At the start of the treatment period the *Datura* plants were at the flowering stage of growth and were sixty-three-day-old seedlings which had been grown in hydroponic culture for twenty-three days. Likewise, the tomato plants had reached the flowering stage and were forty-seven-day-old seedlings which had been grown in hydroponic culture for twenty-four days. Forty-four plants of each species were divided into 12 sets of two or four plants. Half of the plants were harvested at the end of three days of treatment; the remainder at the sixth day. All plants were grown in 1 L. of culture medium.

The concentration of chemicals in the media during the treatment period was one-half that employed during the growth period. Those plants treated with atropine had the designated concentration (0.25 or 0.1%) of atropine sulfate dissolved in the Hoagland's solution. The plants treated with 2,4-D were removed to another room at zero time and the leaves and tops of the plants were thoroughly sprayed with a 0.01% 2,4-D solution. After the spraying period they were brought into the greenhouse and separated from those plants not receiving a 2,4-D spray treatment. Since the desired response from 2,4-D had not been attained with tomato at the end of seventy-two hours, these were further treated at that time by the application of two drops of a 0.1% 2,4-D sodium solution to the growing tips of each plant.

The culture solutions for all plants were then changed every forty-eight hours. The volumes of the used culture media were measured and 30 ml. aliquots were saved from those containing atropine. One-tenth ml. of formalin was added to each aliquot and these were placed in 30-ml. bottles for storage

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in a refrigerator until subsequent analysis for atropine.

Plant Responses Induced by the Various Treatments.—Characteristic responses were induced in both *Datura* and tomato by the six-day treatment with 0.25% atropine. These consisted of severe leaf wilt and droopiness, leaf roughening, loss of lower leaves, and darkening of the roots. White root tips were absent. The degree of injury was much less pronounced at the 0.1% atropine concentration, suggesting that the amount of damage was related to concentration. The atropine effect appeared to be more severe in the leaves and tops of tomato. Further, an additional atropine effect appeared in tomato. This manifested itself as a characteristic necrotic condition of the leaf tissue. Oval darkened areas varying in diameter from 0.1 to 2.0 mm. appeared on many of the larger leaves as well as on some of the stems of tomato.

Stem epinasty, leaf curl, leaf roughening, and in some isolated cases, some stem swelling and blanching were observed. The toxic effects of 2,4-D on the leaves and stems of *Datura* in general were similar to those previously reported by Tsao and Youngken (2) and Youngken (4).

In general, both types of injury, though less extensive, were induced in *Datura* and tomato by the combined 2,4-D and atropine treatment. Although the 2,4-D effect was the first to be observed (within twenty hours), the atropine effect (noted at about thirty-six hours) was much more pronounced after three days in the case of plants receiving the higher concentrations of atropine. At the lower concentration of atropine, the 2,4-D toxic effect was more pronounced.

Correlation of Dry Weight Data.—One series of plants was harvested at three days (seventy-two hours) and the other series at six days (one hundred forty-four hours) after treatment. At each harvest the individual plant was separated into leaves and tops, stems, and root portions. Each portion was then placed in an automatic forced-air drier and dried at a temperature of 42°. The leaf and root portions were allowed to remain at this temperature for thirty-six hours and the stem portions for seventy-two hours. The plant parts were removed from the oven and allowed to attain room temperature before being weighed. The weights of each plant part were recorded and the materials were stored in airtight containers until subsequent pulverization in a Wiley mill. Differences in the dry weight of the plant parts were noted as an indication of comparative growth (Tables I and II).

Alkaloid Analysis for Loss of Atropine from Culture Solution.—The 30-ml. aliquots of the hydroponic culture solutions from the plants treated with atropine were assayed by a modified U. S. P. XIV method (9). The modification consisted of making the 30-ml. aliquots acid by addition of 0.2–0.5 ml. of concentrated sulfuric acid; filtering the solution

TABLE I.—DRY WEIGHTS OF DATURA STRAMONIUM

Treatment	Total Weight Gm.	Total Weight Difference, %	Tops and Leaves Gm.	Tops and Leaves Difference, %
A. Three-day set				
1. Control	10.94 ± 0.53	...	5.23 ± 0.44	...
2. 2,4-D 0.01%	8.76 ± 0.84	−20.0	3.81 ± 0.35	−27.2
3. Atropine 0.1%	6.78 ± 0.89	−38.0	3.11 ± 0.43	−40.5
4. Atropine 0.25%	10.74 ± 0.37	− 1.8	4.51 ± 0.13	−13.8
5. Atropine 0.1% + 2,4-D 0.01%	6.63 ± 0.21	−39.4	2.84 ± 0.20	−45.7
6. Atropine 0.25% + 2,4-D 0.01%	7.91 ± 0.96	−27.7	3.41 ± 0.36	−34.8
B. Six-day set				
1. Control	13.90 ± 0.09	...	6.50 ± 0.15	...
2. 2,4-D 0.01%	11.54 ± 1.84	−17.0	5.01 ± 0.70	−22.9
3. Atropine 0.1%	9.02 ± 0.77	−35.1	3.94 ± 0.32	−39.4
4. Atropine 0.25%	8.90 ± 0.70	−36.0	3.79 ± 0.21	−41.7
5. Atropine 0.1% + 2,4-D 0.01%	7.18 ± 1.00	−48.3	3.01 ± 0.44	−53.7
6. Atropine 0.25% + 2,4-D 0.01%	6.67 ± 0.40	−52.0	2.83 ± 0.26	−56.5

TABLE II.—DRY WEIGHTS OF TOMATO

Treatment	Total Weight Gm.	Total Weight Difference, %	Tops and Leaves Gm.	Tops and Leaves Difference, %
A. Three-day set				
1. Control	8.19 ± 1.78	...	4.58 ± 0.93	...
2. 2,4-D 0.01%	8.77 ± 1.30	7.1	4.75 ± 0.72	3.7
3. Atropine 0.1%	8.76 ± 0.24	6.9	4.54 ± 0.15	−0.9
4. Atropine 0.25%	7.52 ± 0.25	−8.2	3.89 ± 0.14	−15.1
5. Atropine 0.1% + 2,4-D 0.01%	7.50 ± 0.77	−8.4	3.89 ± 0.42	−15.1
6. Atropine 0.25% + 2,4-D 0.01%	7.95 ± 0.57	−2.9	4.03 ± 0.40	−12.0
B. Six-day set				
1. Control	10.02 ± 0.81	...	5.42 ± 0.20	...
2. 2,4-D 0.01%	10.66 ± 0.94	6.4	5.26 ± 0.49	−2.9
3. Atropine 0.1%	9.93 ± 1.12	−0.9	5.16 ± 0.63	−4.8
4. Atropine 0.25%	10.47 ± 1.50	4.5	5.22 ± 0.82	−3.7
5. Atropine 0.1% + 2,4-D 0.01%	11.50 ± 0.96	14.8	5.63 ± 0.65	3.9
6. Atropine 0.25% + 2,4-D 0.01%	9.09 ± 0.68	−9.3	4.27 ± 0.39	−21.2

to remove root particles and other undesirable matter; and then proceeding according to the official method. From these analyses the total milligrams of atropine remaining in the culture solution were subtracted from 2,082 mg. of atropine (equivalent to 2,500 mg. of atropine sulfate) for the sets treated with 0.25% atropine, or from 833 mg. of atropine (equivalent to 1,000 mg. of atropine sulfate) for the sets treated with 0.1% atropine. This was recorded and the data made cumulative to show the total atropine loss from solution (Figs. 1 and 2).

It should not be construed that the total atropine lost from the culture media was taken up through the roots and into the plant. Some atropine loss could have occurred from hydrolysis or degradation of the alkaloid in solution through the action of microorganisms or from other factors. In order to correct for such a loss (which will be termed spontaneous decomposition), four control bottles each containing atropine in concentrations of 0.25% and 0.1%, but without plants, were analyzed periodically for atropine (Figs. 1 and 2).

The use of the spontaneous decomposition factor to calculate absorption was considered satisfactory since the absorption figures generally indicated the trends in uptake in spite of the fluctuations. The correction for spontaneous decomposition has its limitations however, since the rate of degradation in a solution without roots might not be the same as that with the roots of a living plant. Nevertheless, in the absence of more accurate comparative data, this factor proved helpful.

In general, the rate of atropine uptake in the case of plants treated with atropine alone was rapid at first but slow during the latter part of the treatment. The rate of uptake for the sets receiving the combined treatment was slow at the start, increasing more steadily toward the end of the six-day treatment. This phenomenon was much less pronounced in tomato.

Analysis of Total Alkaloids in Leaves and Tops.—The method of Witt, *et al.* (10), was employed with some modification for alkaloid extractions and analyses. The Witt method was modified by using chloroform as the immiscible solvent for extraction instead of benzene since preliminary work showed this to be a more efficient method. The results of the alkaloid analyses are shown in Tables III and IV.

With the exception of the 2,4-D treated set, significant increases in alkaloids were noted for all the six-day sets. As shown in Table III, the increases were from 935 to 4,064%. The 11.8% increase for the set receiving a 2,4-D treatment was considered large, but not significant. The sets treated with atropine alone had a higher percentage increase at both atropine concentrations than the sets receiving the combined treatment. The results of the analysis for alkaloids in tomato leaves and tops are shown in Table IV. All tomato sets treated with atropine accumulated a significant amount of alkaloid. The increases for the six-day tomato sets varied from 4,180 to 22,300%. However, when six-day sets receiving the combined 2,4-D and atropine treatment were compared with those treated only with atropine, inconsistent results were observed.

It was further desired to make comparisons based on the total alkaloids per plant leaves and tops

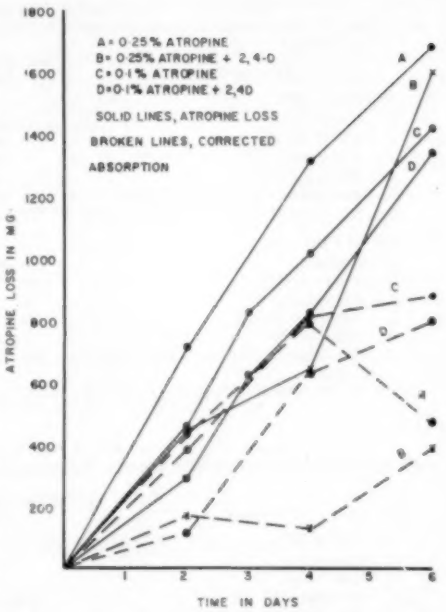
A = 0·25% ATROPINE
B = 0·25% ATROPINE + 2, 4-D
C = 0·1% ATROPINE
D = 0·1% ATROPINE + 2,4D
SOLID LINES, ATROPINE LOSS
BROKEN LINES, CORRECTED
ABSORPTION
ATROPINE LOSS IN MG.
TIME IN DAYS

Fig. 1.—Total atropine loss from culture solution in *Datura stramonium* (average per combined 3- and 6-day sets).

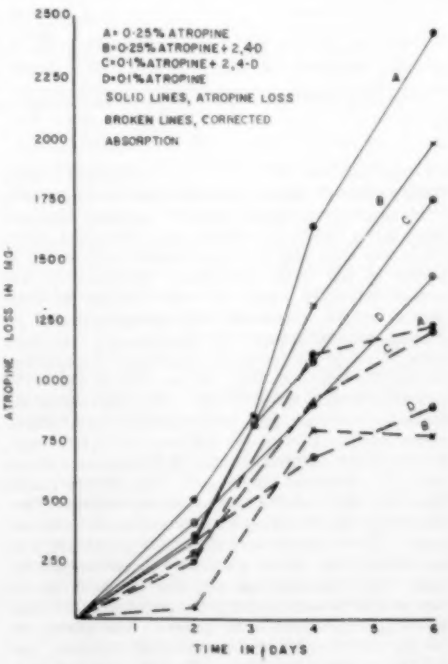
A = 0·25% ATROPINE
B = 0·25% ATROPINE + 2,4-D
C = 0·1% ATROPINE + 2, 4-D
D = 0·1% ATROPINE
SOLID LINES, ATROPINE LOSS
BROKEN LINES, CORRECTED
ABSORPTION
ATROPINE LOSS IN MG.
TIME IN DAYS

Fig. 2.—Total atropine loss from culture solution in tomato (average per combined 3- and 6-day sets).

TABLE III.—ANALYSIS OF ALKALOIDS IN DATURA STRAMONIUM LEAVES

Treatment	(A) Total Alkaloids Calcd. as Hyoscyamine mg./Gm.	(A) Difference, %	(B) Total Leaf Alkaloids mg./pl.[a]	(B) Difference, %
A. Three-day set				
1. Control	0.58 ± 0.04	...	3.02	...
2. 2,4-D 0.01%	0.58 ± 0.04	0.0	2.20	−27.1
3. Atropine 0.1%	5.48 ± 0.50	845.0	16.49	446.0
4. Atropine 0.25%	14.44 ± 0.48	2,390.0	64.97	2,052.0
5. Atropine 0.1% + 2,4-D 0.01%	5.40 ± 0.72	831.0	15.21	404.0
6. Atropine 0.25% + 2,4-D 0.01%	13.45 ± 2.00	2,217.0	48.14	1,494.0
B. Six-day set				
1. Control	0.68 ± 0.16	...	4.42	...
2. 2,4-D 0.01%	0.76 ± 0.04	11.8	3.79	−14.3
3. Atropine 0.1%	8.52 ± 1.32	1,153.0	34.47	680.0
4. Atropine 0.25%	29.38 ± 3.0	4,064.0	112.0	2,433.0
5. Atropine 0.1% + 2,4-D 0.01%	7.04 ± 0.64	935.0	22.03	397.0
6. Atropine 0.25% + 2,4-D 0.01%	14.42 ± 1.01	2,020.0	41.22	833.0

[a] Obtained by calculation from dry weight and alkaloid analyses data.

TABLE IV.—ANALYSIS OF THE ALKALOIDS IN TOMATO LEAVES AND TOPS

Treatment	(A) Total Alkaloids Calcd. as Hyoscyamine mg./Gm.	(A) Difference, %	(B) Total Leaf Alkaloids mg./pl.[a]	(B) Difference, %
A. Three-day set				
1. Control	0.12	...	0.44	...
2. 2,4-D 0.01%	0.12	0.0	0.60	36.3
3. Atropine 0.1%	4.66 ± 0.48	3,783.0	21.24	5,253.0
4. Atropine 0.25%	12.18 ± 0.12	10,040.0	47.37	10,620.0
5. Atropine 0.1% + 2,4-D 0.01%	3.02 ± 0.28	2,417.0	11.41	2,493.0
6. Atropine 0.25% + 2,4-D 0.01%	9.60 ± 0.54	7,900.0	39.05	8,774.0
B. Six-day set				
1. Control	0.10	...	0.56	...
2. 2,4-D 0.01%	0.14	40.0	0.78	41.0
3. Atropine 0.1%	5.54 ± 0.64	5,440.0	29.33	5,203.0
4. Atropine 0.25%	16.06 ± 2.60	15,960.0	88.28	15,860.0
5. Atropine 0.1% + 2,4-D 0.01%	4.28 ± 0.34	4,180.0	23.41	4,133.0
6. Atropine 0.25% + 2,4-D 0.01%	22.40 ± 5.6	22,300.0	99.44	17,880.0

[a] Obtained by calculation from dry weight and alkaloid analyses data.

(Tables III and IV). The total alkaloids (calculated as hyoscyamine) were obtained by calculation from the dry weight and alkaloid analyses data and stating the results in milligrams. When the above basis was used and the comparisons were stated in terms of per cent, significant increases in leaf accumulation of alkaloids were induced in both species by the treatments with atropine alone. In *Datura*, the increases for the six-day sets, compared with those of the controls, amounted to approximately 2,433% and 680% at the 0.25% and 0.1% concentrations, respectively. The corresponding increases in tomato were about 15,800% and 5,200% respectively. Under the influence of 2,4-D only, *Datura* plants showed about a 14.0% decrease which was not considered significant. The *Datura* plants receiving the combined treatment, likewise, displayed significant alkaloid increases per plant leaves-tops. The increases were about 833% and 397% at the higher and lower alkaloid concentrations, respectively. Correspondingly, the increases in the case of the tomato plants were about 17,800% and 4,100%, respectively. In general, the plants receiving the combined treatment of atropine and 2,4-D accumulated less alkaloids than plants treated with atropine alone.

SUMMARY

Toxic concentrations of atropine and 2,4-dichlorophenoxyacetic acid were administered to *Datura stramonium* and *Lycopersicon esculentum*, and effects on alkaloid accumulations in both plants are described. The atropine in concentrations of 0.25% and 0.1% was added to the culture media and the 2,4-D (0.01% solution) was sprayed on the leaves. The following salient points were observed:

1. Characteristic toxic responses in growth were induced in both species by the treatment with atropine, 2,4-D, and a combination of the two chemicals.

2. There was a significant increase in the leaf accumulation of atropine-type alkaloids under the conditions of the experiment. The increases per plant tops-leaves at the higher atropine concentration were approximately 24-fold in *Datura* and 158-fold in tomato. Correspondingly, the increases at the lower concentration were 7-fold and

52-fold. In *Datura*, treatment with a combination of atropine and 2,4-D resulted in a significant decrease in leaf alkaloid when compared with the treatment during which atropine alone was used. The decrease in atropine accumulation was attributed to the influence of 2,4-D.

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Preservative Action of Combined Bacteriostatic Agents I.*

Preservative Action of Chlorobutanol in Combinations with Certain Other Bacteriostatic Agents

By EDWARD N. DEEB and JOHN W. BOENIGK

Chlorobutanol was found to be readily soluble in Tween[1] 20, benzyl alcohol, and phenylethyl alcohol. The concentrated solutions are easily diluted to 0.5 per cent aqueous solutions, which is the strength frequently used in ophthalmic solutions. The bacteriostatic and bactericidal efficiency of the combined agents was found to be greater than that of chlorobutanol 0.5 per cent in isotonic sodium chloride solution.

A REVIEW of the literature regarding the preservative action of various chemicals indicated that relatively little work has been reported on the use of combined bacteriostatic agents for ophthalmic solutions (1). It seemed possible that some degree of synergism might exist between certain antibacterial agents. Because chlorobutanol is one of the two most widely used preservative agents at the present time, combinations of this agent with benzyl and phenylethyl alcohols were selected for evaluation in this study.

Chlorobutanol has been used as a preservative for ophthalmic solutions for a number of years (2). It has the disadvantage that it is slightly and slowly soluble in water. The solubility of chlorobutanol is listed in the U. S. P. as 1 Gm. in 125 ml. of distilled water (0.8 per cent). It was determined experimentally that one hour and twenty minutes of constant stirring in a magnetic stirrer were required to dissolve 0.5 Gm. of anhydrous chlorobutanol U. S. P. in 100 Gm. distilled water at room temperature. Chlorobutanol is more soluble in boiling water; however, such solutions have diminished preservative action probably due to loss by volatilization and hydrolysis (2).

In order that preservative activity of solutions used in this study would not be misinterpreted as being due to hydrogen ion concentration, the pH value of the more important solutions used in this study were determined and it was found that none of the solutions had a pH lower than 4.3. These determinations were considered pertinent because Fredenburgh and Heicht (3) found that hydrogen ion concentration corresponding to pH 4.1 or lower inhibited the growth of pathogenic bacteria.

Phenylethyl alcohol has been reported to have antibacterial action by Brewer (4), Grote (5), and Lilley (6). The value of benzyl alcohol as a preservative in ophthalmic solutions has been reported by Gershenfeld (7).

EXPERIMENTAL

Chlorobutanol Concentrate with Tween 20.—Due to the inherent difficulties experienced with extemporaneous solutions of chlorobutanol in water or normal saline, a study was made to determine if a concentrated solution of chlorobutanol could be prepared. Spans[1] and polyvinylpyrrolidone proved to be unsuitable as solubilizing agents. Of the series of Tweens tried, Tween 20 proved to be the best solubilizing agent for chlorobutanol. A 50% solution in Tween 20 was possible; however, all so-

* Received April 25, 1958, from The Veterans Administration Hospital, Pittsburgh, Pa., and the University of Pittsburgh, School of Pharmacy, Pittsburgh, Pa.
[1] Registered trademark name of Atlas Powder Co.

lutions greater than 33% precipitated chlorobutanol when diluted to a 0.5% concentration. A 10% solution of chlorobutanol was chosen as the most convenient concentrate to use. This solution was prepared by adding 10 Gm. of chlorobutanol to 40 Gm. of Tween 20, stirring the mixture in a magnetic stirrer until solution was complete, at which time the remaining 50 Gm. of sterile distilled water was added.

A 0.5% solution prepared from the 10% concentrate in Tween 20 was tested for possible irritation and sensitivity. It was found that the solution did not cause any more irritation than chlorobutanol 0.5% in isotonic sodium chloride solution. The irritation and sensitivity tests were made on the eyes of seven rabbits. Three drops[2] of a 0.5% solution prepared from the Tween 20 concentrate were placed in one eye and a control solution of 0.5% of chlorobutanol in isotonic sodium chloride solution was placed in the other eye. These solutions were placed in the rabbits eyes three times a week for two weeks. No apparent sensitivity was observed. The results of these tests were observed by Dr. Lehman.[3]

Chlorobutanol with Benzyl and Phenylethyl Alcohols.—Experimentation showed that chlorobutanol was freely soluble in both benzyl and phenylethyl alcohol, and that a 50% solution in either alcohol could be made. Both benzyl alcohol (7) and phenylethyl alcohol (4–6) have been used as preservatives in ophthalmic solutions with no reported unusual irritation or sensitivity. Concentrated solutions of chlorobutanol in benzyl and in phenylethyl alcohol can be readily diluted to 0.5% strength, although the chlorobutanol–benzyl alcohol concentrate must be diluted by gradual addition to water or isotonic sodium chloride solution. The diluted solutions containing chlorobutanol and benzyl alcohol 0.5% each, and chlorobutanol and phenylethyl alcohol 0.5% each, were tested for irritability and sensitivity as described above, using the same control. The combination of chlorobutanol and phenylethyl alcohol was found to be slightly more irritating than the control. Neither solution appeared to cause any sensitivity.

Preservative Activity Studies.—The preservative properties of the solutions prepared were tested against organisms which are pathogenic to the eye (8) and which have been reported to occur in ophthalmic solutions (9).

Chlorobutanol Concentrate in Tween 20.—All organisms used in testing for preservative activity were isolated from patients at this hospital.[4] All organisms were pathogenic and resistant to one or more antibiotics.

Solutions of various percentage strengths of chlorobutanol were prepared by dilution of the 10% chlorobutanol-Tween 20 concentrate with Brain Heart Infusion broth (BHI). Five-ml. portions of these solutions were inoculated with 0.05 ml.[5] of a twenty-four-hour BHI broth culture of the bacterial organisms. At the same time, solutions of the same concentrations were made by dissolving chlorobutanol in BHI broth without the addition of any Tween 20, and these solutions were inoculated with the same organisms. All test solutions were incubated at 37°. The cultures were observed at twenty-four-hour intervals. Growth in BHI broth in nearly all tests was easily observed by macroscopic examination.

The results of the preservative action of chlorobutanol in the two media are presented in Table I. Solutions of chlorobutanol containing Tween 20 had less preservative action than solutions of chlorobutanol alone.

Combinations of Chlorobutanol with Benzyl Alcohol and with Phenylethyl Alcohol.—The con-

TABLE I.—BACTERIOSTATIC EFFECT OF CHLOROBUTANOL IN BRAIN HEART INFUSION BROTH WITH AND WITHOUT TWEEN 20

Organism Strain		Chlorobutanol Concentration in Solution, % 0.75	0.5	0.4	0.3	0.2
Ps-1	T	+	+	+	+	+
	A		−	−	−	+
Ps-2	T	−	−	+	+	+
	A		−	−	+	+
Ps-3	T		−	−	−	+
	A		−	−	−	+
Ps-4	T		+	+	+	+
	A		−	+	+	+
Ps-5	T		−	−	−	−
	A		−	−	−	−
S-1	T	−	−	−	−	+
	A		−	−	−	+
S-2	T	−	−	−	+	+
	A		−	−	−	+
S-3	T	−	−	−	−	+
	A		−	−	−	+
Prot-1	T	−	−	−	+[a]	+
	A		−	−	−	+

Ps—Pseudomonas aeruginosa, S—Staphylococcus aureus, Prot—Proteus vulgaris, T—Tween 20 added, A—without Tween 20.
− No growth after 72 hours' incubation. + Growth after 24 hours' incubation.
+[a] Growth after 48 hours' incubation. All control tubes showed growth.

TABLE II.—PRESERVATIVE ACTIVITY OF COMBINATIONS OF BACTERIOSTATIC AGENTS IN BRAIN HEART INFUSION BROTH

Organism Strain	Results after 72 Hours' Incubation: Chlorobutanol-Benzyl Alcohol, of each, % 0.5	0.4	0.3	0.2	Chlorobutanol-Phenylethyl Alcohol, of each, % 0.5	0.4	0.3	0.2
Ps-1	−	−	−	−	−	−	−	+
Ps-2	−	−	−	−	−	−	−	+
Ps-3	−	−	−	−	−	−	−	−
Ps-4	−	−	+	+	−	−	−	+
Ps-5	−	−	−	−	−	−	−	−
Prot-1	−	−	−	−	−	−	−	−

All controls showed growth after 24 hours' incubation.
Ps—Pseudomonas aeruginosa, Prot—Proteus vulgaris.

[2] San-I-Drop, sterile disposable plastic medicine droppers of Marnel Incorporated, Alexandria, Va., were used to instill the eye solution.

[3] The authors wish to thank Dr. Robert N. Lehman, Associate Professor of Ophthalmology at the University of Pittsburgh, School of Medicine, and Chief, Ophthalmology Service at this hospital, for the external examination of the conjunctiva of the rabbits following instillation of the eye solutions and controls.

[4] The authors are indebted to James V. Datillo, Chief Bacteriologist of this hospital for the isolation and identification of the organisms used in this study, his technical assistance in setting up the testing program, and interpretation of the results of the bacteriological tests.

[5] The average of five counts showed that 0.05 ml. of one of the twenty-four-hour cultures of Pseudomonas contained 35,500,000 organisms.

TABLE III.—THE RELATIVE BACTERIOSTATIC EFFICIENCY OF INDIVIDUAL PRESERVATIVES INVESTIGATED
First Appearance of No Growth in Subculture Tubes (in Hours)

Organism Strain	Chlorobutanol 0.5%	Benzyl-Alcohol, 0.5%	Phenylethyl Alcohol, 0.5%	Chlorobutanol-Benzyl Alcohol of each, %				Chlorobutanol-Phenylethyl Alcohol of each, %			
				0.5	0.4	0.3	0.2	0.5	0.4	0.3	0.2
Ps-1	+24	+24	+24	−1	−4	+4	+4	−1	−1	+4	+4
Ps-2	−24	+24	−24	−1	−4	+4	+4	−4	−4	+4	+4
Ps-3	−1	−24	−24	−1	−1	−1	+4	−1	−1	−1	+4
Ps-4	−24	+24	−24	−24	−24	+24	+4	−1	−4	+4	+4
Ps-5	−1	−24	−4	−1	−1	−1	−4	−1	−1	−4	+4
Ps-6	+24	−24	−4	−1	−4	+4	+4	−1	−1	−1	+4
S-1	−24	+24	+24	−1	−4	+4	+4	−1	−1	−4	+4
S-2	−24	+24	−24	−1	−1	+4	+4	−1	−1	−4	+4
S-3	−24	+24	−24	−1	−1	+4	+4	−1	−1	+4	+4
Prot-1	−24	+24	−24	−1	−1	−1	+4	−1	−1	−4	+4

Ps—*Pseudomonas aeruginosa*, S—*Staphylococcus aureus*, Prot—*Proteus vulgaris*.
+4—Indicates viable organisms in subculture made four hours after initial inoculation.
−4—Indicates no growth in subculture made four hours after initial inoculation.
Each of the above preservative agents was dissolved in isotonic sodium chloride solution containing 1% atropine sulfate.
Each of the subculture tubes was inoculated with 0.5 ml. of the experimentally contaminated preservative solutions mentioned in the above table.

centrates of chlorobutanol in benzyl alcohol and in phenylethyl alcohol were diluted to 0.5% concentration of each chemical with sterile BHI broth and the preservative properties of the combinations were tested by culturing them with five Pseudomonas and one Proteus organisms (The Pseudomonas organisms were the same five used to test the chlorobutanol and the chlorobutanol-Tween 20 solutions, Table I). The limited testing in BHI broth, as presented in Table II, demonstrated that both of the combination preservative solutions were more effective than chlorobutanol alone. No further tests were made in BHI broth with any other organisms since it was considered of greater value to determine preservative efficiency in an aqueous medium, the normal medium of ophthalmic solutions.

Lawrence (10) reported that the actions of many preservative agents were enhanced by the presence of drugs commonly used in ophthalmic solutions. A third series of tests was made to determine if the combination solutions of chlorobutanol and phenylethyl alcohol would have increased activity in the presence of a commonly used ophthalmic drug. A 1% solution of atropine sulfate was chosen as a typical ophthalmic solution. The results of this test series are reported in Table III. The combination of chlorobutanol with phenylethyl alcohol was found to be more effective than the combination of chlorobutanol with benzyl alcohol. There was slight but inconclusive evidence that the solutions containing the atropine sulfate were more efficient preservatives than those without the atropine sulfate.

SUMMARY AND CONCLUSIONS

1. A concentrated solution of chlorobutanol can be prepared in Tween 20, and the concentrate is readily diluted with water or isotonic sodium chloride solution.

2. Chlorobutanol is readily soluble in benzyl alcohol and phenylethyl alcohol, and the concentrated solutions of these combined preservatives can be readily diluted with water or isotonic sodium chloride solution.

3. Aqueous solutions containing the combined preservatives, chlorobutanol with benzyl alcohol, and chlorobutanol with phenylethyl alcohol 0.5 per cent each have a greater preservative action than a 0.5 per cent aqueous solution of chlorobutanol. The results of the tests suggest synergism between the combined preservatives and indicate that the mechanism of bacteriostatic action of chlorobutanol differs from that of either benzyl or phenylethyl alcohol.

4. A 0.75 per cent chlorobutanol solution with Tween 20 has less preservative activity than a 0.5 per cent solution without Tween 20. This may be seen by a study of Table I.

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The Effect of Hydrogen Ion and Alcohol Concentration on the Solubility of Phenobarbital*

By T. D. EDMONSON and J. E. GOYAN

The solubility of phenobarbital in alcohol with varying pH has been determined. The data should make it possible to determine the approximate solubility of phenobarbital for any pH and any alcohol concentration up to 50 per cent. The data indicate that it is possible to prepare phenobarbital solutions which have both good stability and solubility using minimal amounts of alcohol if the pH is adjusted.

MANY of the medicinal agents currently employed in medical practice are either weak organic acids or bases. Since they are relatively insoluble in water, their salts are employed to prepare solutions of effective therapeutic concentration. In certain instances, however, the use of the salt may decrease the stability appreciably. One such group of drugs is the barbiturates.

It is well known that the salts of the barbiturates decompose when dispersed in aqueous solutions (1). The stability, however, may be increased if the pH of the solution is decreased. Unfortunately, the reduction of pH soon reaches a minimum value, where the concentration of the free acid formed is greater than its aqueous solubility. This unfortunate situation of "good stability" and "poor solubility" has prompted those in the pharmaceutical field to search for other methods of solubilizing the acidic form of the drug.

The utilization of solubilizing agents such as the polyoxyethylene sorbitan esters has met with some success (2), but the objectionable taste has prevented wider use of these agents in oral formulations of the barbiturates.

Solvents or combinations of solvents such as glycerin-alcohol-water, propylene glycol-water, and alcohol-water mixtures have all been suggested for the preparation of suitable barbiturate preparations (3–5). Still another approach has been the use of water-alcohol mixtures with proper pH adjustment of the existing solution. In this manner an optimum pH and alcohol concentration could be achieved which would produce sufficient solubility and stability.

Urdang and Leuallen (6) have reported a study of the solubility of phenobarbital as a function of pH. Repetition of their experiments has shown an appreciable change in pH upon dissolution of the acidic phenobarbital. The solvent at pH 8.0, for example, after saturation with phenobarbital, was found to have a pH of 7.5.[1]

Actually at pH 8.0 the buffer is approximately 0.02 *M* in Na_2CO_3 and 0.18 *M* in boric acid. The phenobarbital concentration is about 0.03 *M*. The buffer capacity would therefore seem to be inadequate.

In order to verify and correct existing solubility data on phenobarbital as a function of pH and alcohol concentration, this study was initiated.

EXPERIMENTAL

Materials and Apparatus.—U. S. P. grades of phenobarbital and sodium phenobarbital were used. All other chemicals were reagent grade. A Beckman model DU spectrophotometer and a Leeds and Northrup model 7664 pH meter were used.

Assay.—The absorbance spectra of phenobarbital were determined in three different solutions. Each solution consisted of 10^{-4} moles of recrystallized phenobarbital dissolved in one liter of the solvent. Solvent I was distilled water, solvent II a dilute buffer solution with a pH of 9.91, solvent III a 4.6 *M* sodium hydroxide solution. The spectra are shown in Fig. 1. The reason for determining these three spectra are explained under discussion. The two absorbance maxima at 240 and 255 mμ were used in our analysis.

An aliquot of the phenobarbital solution was diluted with a solution having a pH greater than 10 (in order to limit the ionic forms to Pb^- and $Pb^=$), and then the absorbance of the solution determined at 240 and 255 mμ. The concentration of phenobarbital could then be calculated by using simultaneous equations (7).

Solubility of Phenobarbital as a Function of Alcohol Concentration.—In the first series of experiments, excess phenobarbital was added to approximately 25 ml. of various concentrations of alcohol-water mixtures in 50-ml. Erlenmeyer flasks. The flasks were then stoppered with cork wrapped in Parafilm[2] and placed into a shaking apparatus con-

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Presented at the American Pharmaceutical Association Convention, Los Angeles, Calif., April 21, 1958.

[1] Since most pharmaceutical vehicles also have a low buffer capacity, their solubilities may be close to those in such vehicles.

[2] Trademarked product of Marathon Corp., Menasha, Wis.

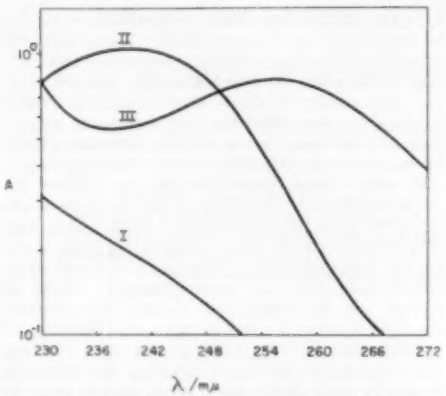

Fig. 1.—Ultraviolet absorption spectra of the different ionic species of phenobarbital.

tained in a constant temperature water bath maintained at a temperature of 25° ± 0.1°.

After forty-eight hours of agitation, each solution was filtered and a one-ml. aliquot introduced into a 1,000-ml. volumetric flask. Twenty-ml. of a 0.2 *M* sodium hydroxide solution and enough distilled water were added to bring the volume up to 1,000 ml. The solubility of the phenobarbital at each alcohol concentration was then determined according to the spectrophotometric method described under "Assay." Table I includes these results.

Solubility of Phenobarbital as a Function of pH and Alcohol Concentration.—The pKa values of phenobarbital at several alcohol concentrations were previously determined in the laboratory. A plot of the reciprocal of the dielectric constant against pKa produced a linear relationship up to an alcohol concentration of 60%, as predicted by electrostatic theory (8). Figure 2 shows this relationship. From the figure it is possible to determine the pKa of phenobarbital at any alcohol concentration.

It is evident that by the use of the buffer equation:

$$pH = pKa + \log \frac{Salt}{Acid}$$

the approximate concentration of the salt of phenobarbital may be calculated for any desired pH, phenobarbital concentration, and alcohol concentration. The pKa values and acid solubility may be obtained from Table I and Fig. 2.

A number of solutions were prepared in 50-ml. flasks at a definite pH and alcohol concentration. The quantity of salt necessary for each pH and alcohol concentration was first calculated and added to the proper flasks. To each flask an excess of the free acid was also added and these flasks placed into the shaking apparatus, as previously described, and agitated for twenty-four hours. Each flask was then removed and the pH adjusted to the exact pH desired with 1.0 *M* HCl. or 1.0 *M* NaOH. Since the volume of base or acid added was very minute, it did not appreciably alter the concentration of the alcohol. The flasks were returned to the shaker and agitated for a period of twenty-four hours. After equilibrium had been reached, the solutions were assayed for their phenobarbital content. The solubility results are included in Table I.

TABLE I.—SOLUBILITY GM./100 cc.

Alcohol, %	pKa[a]	H_2O	pH 7.0	pH 8.0
0	7.42	0.13	0.18	0.72
5	7.48	0.15	0.21	0.83
10	7.58	0.18	0.24	0.93
15	7.64	0.23	0.30	1.04
20	7.74	0.34	0.37	1.19
25	7.82	0.44	0.48	1.37
30	7.92	0.58	0.67	1.60
35	8.04	0.94	0.97	1.88
40	8.16	1.36	1.42	2.30
45	8.30	2.02	2.03	2.76
50	8.48	2.88	2.89	3.47

[a] The pKa values were read off a graph of pKa *vs.* 1/dielectric constant which gives a straight line relationship (Fig. 2).

DISCUSSION

The assay procedure we followed differs from that used by Urdang and Leuallen (6). They diluted their sample aliquots with the appropriate buffer solution and used an absorbance index determined for each pH. It was felt that this method has some disadvantages. This can be shown by noting the following equations:

$$H_2O + Pb = Pb^- + H_3O^+$$
$$H_2O + Pb^- = Pb^= + H_3O^+$$

Solutions I, II, and III on Fig. 1 correspond closely to these three forms. This shows that the sensitivity of the method changes greatly with pH. Indeed at lower pH values no maximum exists at 240 mμ.

The spectra of the water and sodium hydroxide solutions give the desired absorbance maxima A_m for Pb and $Pb^=$ directly. However the buffer solution contains small amounts of Pb and $Pb^=$ in addition to the Pb^-. This was corrected for by using the following equations, modified from Deford (9).

$$\frac{Pb}{Pb^=} = \frac{(H_3O^+)^2}{K_1K_2},$$

$$Pb^- = \frac{\text{Total phenobarbital added } (10^{-4}\ M)}{\frac{H_3O^+}{K_1} + \frac{K_2}{H_3O^+} + 1}$$

The value of K_2 was adapted from Butler, *et al.* (10). Activity effects were neglected since they should be negligible at low concentrations.

Now the total absorbance at any wavelength should be:

$$A_R = a_1Pb + a_2Pb^- + a_3Pb^=$$

We can now calculate Pb, Pb^-, and $Pb^=$ and knowing A_R, a_1, and a_3, we can solve for a_2. This was done at 240 and 255 mμ. The values are a_{240} = 10,800 liters moles^{-1} cm.$^{-1}$ and a_{255} = 4,340 liters moles^{-1} cm.$^{-1}$.

The validity of these values was checked by using them to calculate what the $a_{240,1\%}$ should be for a phenobarbital solution with a pH = 9.5. This gave a value of 459 which is in exact agreement with the value for the same constant given by Mattson (11).

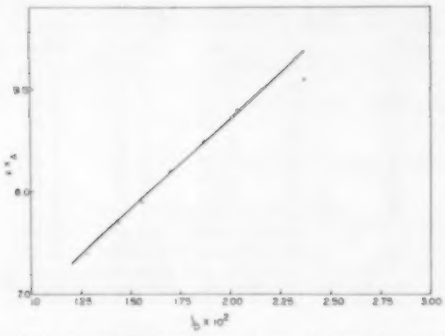

Fig. 2.—Dissociation constant of phenobarbital as a function of dielectric constant. The points correspond to 0, 20, 30, 40, 50, 60, and 70% alcohol v/v, respectively.

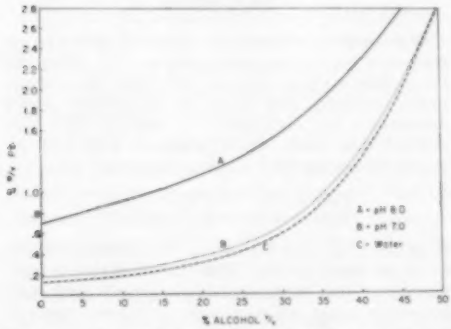

Fig. 3.—The solubility of phenobarbital as a function of alcohol concentration and pH.

The solubility results are shown in Fig. 3. It should be noted that our values for the solubility in water which should agree with Urdang and Leuallen (6) are not in too good agreement. However, it is also true that other values reported by Krause and Cross (3) and Anderson (12) are also at variance, our values falling within the ranges of the other workers.

Our results confirm those of Urdang and Leuallen (6) and others in that we also find it necessary to go to fairly high concentrations of alcohol before the solubility of the free phenobarbital increases significantly. This phenomenon has been reported earlier for other chemicals. It was found for example with acetophenetidin by Gregg-Wilson and Wright (13).

It must be borne in mind that addition of alcohol to water solutions has two effects: It increases the solubility of the free acid and it decreases the ionization of the acid. As pointed out by Higuchi, *et al.* (14), this usually results in a net increase in solubility since the first effect is greater than the second. This is found true in our studies where the solubility increases steadily with alcohol concentration at each pH. These values should also follow the equation developed by Higuchi (15) to describe the change in solubility with pH for a weak acid or base providing we use the pKa corresponding to the given alcohol concentration. This was done and slope values from 1.00–1.20 were obtained. These of course are for only two points but indicate that the results are in line with the theoretical.[1]

Solubilities for pH values greater than 8.0 were not determined experimentally since the solubility is always much larger than would be necessary to provide therapeutic effect in convenient volumes and since this approaches pH values where decomposition might be of importance in prescriptions used over long periods of time.

If the average dose of phenobarbital is taken as 30 mg., this concentration can be obtained with 35% alcohol at pH 7.0 or 10% alcohol at pH 8.0. It would appear, therefore, that a solution of phenobarbital can be made to contain therapeutic amounts at much smaller concentrations of alcohol if the pH is also adjusted.

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[1] Using such plots it is possible to predict the solubility for any pH value at a given alcohol concentration.

Stress Resistance in Thymectomized Rats*

By ALFRED P. IACOBUCCI and HOWARD J. JENKINS

A method for determining stress resistance is employed for the investigation of a possible difference between thymectomized and normal animals with respect to their abilities to withstand the stressing effects of various nonspecific stressor agents. Rats are used as the test animals, and the experimental data evaluated by the Litchfield-Wilcoxon method. Evidence is provided to indicate that there is no significant difference in the ability of normal and thymectomized groups to withstand the stressing effects of dry heat, reserpine, and histamine. The restoration of normal stress resistance in thymectomized, adrenalectomized animals receiving adrenal cortical extracts is not dependent upon the process of thymus involution.

A THOROUGH SEARCH of the literature as well as a personal communciation (1) has revealed that the relationship between the thymus gland and stress resistance has not been clearly set forth.

The purpose of the work of this paper is to provide information which might help to clarify this relationship and, at the same time, to shed some light on the possible role of the mechanism of stress resistance in myasthenia gravis.

In connection with the latter it is known that the thymus gland normally is relatively large at birth, begins to involute at puberty, and is almost completely atrophied at the age of sixty years or so (2). In myasthenia gravis however, this normal involutionary process does not occur in a large proportion of cases.

It has been reported that approximately 55 per cent of all myasthenia gravis patients are found at operation or post-mortem examination to have either a thymoma or else a persistent or hyperplastic thymus gland (3, 4). Moreover, myasthenia gravis patients have a very low resistance to all types of physiologic stress (4). Finally, corticotropin alleviates the symptoms of myasthenia only in individuals who have enlarged thymus glands (5), bringing about as it does, an involution of the gland. In the adrenalectomized animal with a large thymus gland, the administration of adrenal cortical extracts results in the restoration of stress resistance at the same time that the thymus involutes (6).

In view of the foregoing information, the question arises as to the role of the involution of the thymus gland in stress resistance. Is the involution of the enlarged thymus essential to the restoration of normal stress resistance or is it simply a side effect? If it is essential to the restoration of normal stress resistance, and if corticotropin is effective in myasthenia gravis by virtue of its ability to restore resistance through involution of the thymus, then impaired stress resistance is a factor in myasthenia gravis.

EXPERIMENTAL

In an effort to clarify the relationship between the thymus gland and stress resistance, the effects of various stressing agents were measured in both control and thymectomized rats.

This was done by recording the lethal dose for 50% of the animal sample (LD_{50}) for each stressor in a control group and in a thymectomized group. In this manner the effect of thymectomy was determined.

To further clarify the relationship of the thymus to stress resistance, a group of thymectomized, adrenalectomized animals was subjected to a stressor and the resultant LD_{50} compared with the LD_{50} of a control group subjected to the same doses of the same stressor. Then, stress resistance of a similar thymectomized, adrenalectomized group was made equivalent to that of the control group by the administration of adrenal cortical extracts. In this manner, the effect of the process of involution of the thymus gland was determined.

Since this investigation was concerned with nonspecific stress, stressors of various types were selected for the experimental work. Dry heat, one of those selected can be classified as a nondrug, nonspecific stressor. Reserpine was selected as representative of a stressor of long duration, since its peak effect is not reached for a period of about three or four days (7). Histamine was chosen for its ability to initiate stress rapidly and so typify the short-acting agent. Throughout the work, male, albino rats of the Carworth-Wistar strain were used. These animals weighed within the range of 35 to 50 Gm. at the beginning of each study and did not exceed 65 Gm. at its completion. During the entire study the rats were kept in a small, ventilated, temperature-controlled room at 70° F. ±5°. They were maintained on Rockland diet and water, *ad libitum*, the only exception being those thymectomized, adrenalectomized animals which received normal saline instead of tap water.

Dry-Heat Stress.—Twenty rats were thymectomized and, after a forty-eight-hour postoperative re-

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covery period, these rats along with 20 controls were placed in individual wire baskets, which were inverted and arranged in a modified Latin square (7) design on two shelves of an altered incubator. The rats were so distributed that each thymectomized animal was adjacent to a control, both in columns and in rows. The incubator was fitted with a suitable inlet for air. Several thermometers were placed in the incubator at various locations to determine that there was an even distribution of heat in the oven. A small, six-volt, rubber-blade fan was placed on the floor of the incubator, serving to circulate the heat uniformly. The incubator was adjusted to 41° ± 0.2° C. The rats were exposed to this heat for a maximum period of one hundred and twenty minutes. The survival time for each rat was determined by recording the mortality at five-minute intervals. The resultant data were plotted and the median lethal time (LT_{50}) was calculated by the method of Litchfield and Wilcoxon (8).

Reserpine-Induced Stress.—The use of reserpine as a stressor presented a solubility problem, since this agent is insoluble in water and only slightly soluble in nontoxic organic solvents. Propylene glycol was first tried but rejected since its administration resulted in the formation of tumorous growths at the site of injection. The character of these growths was not established since they were not examined for malignancy. Polyethylene glycol ("Carbowax" 200) was effective in solubilizing reserpine to the extent of 1.5 mg./ml. This relatively low solubility necessitated the injection of excessive volumes of solution, however. Serpasil Placebo Vehicle "C"[1] permitted a maximum concentration of reserpine of 2.5 mg./ml., and this vehicle was adopted as the solvent for this portion of the work.

As in the work with dry heat, 30 rats were thymectomized and allowed to recover for a period of forty-eight hours. Reserpine alkaloid, in doses of 25, 50, and 75 mg./Kg. of body weight, was injected intraperitoneally into three groups of ten animals each, and the mortality determined after four days. The same doses were administered in the same manner to a like group of control rats. The data for both groups of animals were then plotted and their median lethal doses calculated after the method of Litchfield and Wilcoxon.

Intraperitoneal injections of 2 ml. of solvent alone in another group of rats seemed to be without biologic activity.

Histamine-Induced Stress.—In the work with histamine phosphate U. S. P., 30 rats were thymectomized and, after a forty-eight-hour recovery period, divided into three like groups of ten animals each. Distilled water was used as the solvent and injections were made intraperitoneally into the lower left quadrant of the abdomen.

The histamine solution volumes were so adjusted that the injections did not exceed 1 ml. Dosage levels of 800 mg./Kg., 1,200 mg./Kg., 1,600 mg./Kg., and 2,000 mg./Kg. were employed.

The experimental procedure was the same as that followed in the work with reserpine, and the median lethal doses were calculated again by the Litchfield and Wilcoxon method.

Histamine-Induced Stress in Thymectomized, Adrenalectomized Rats.—Each of 30 rats was first bilaterally adrenalectomized then thymectomized a short time later, before regaining consciousness. This surgically-treated sample was then maintained on saline and allowed to recover for a period of forty-eight hours, following which it was divided into three groups of ten animals each. The groups were then subjected to dosage levels of 800 mg./Kg., 1,200 mg./Kg., and 1,600 mg./Kg. of histamine phosphate. The number of animals succumbing at each dosage level was determined after forty-eight hours and recorded.

A similar group of thymectomized, adrenalectomized rats was maintained on normal saline for forty-eight hours following surgery, during which time 0.25 mg. of desoxycorticosterone acetate (DOCA) in sesame oil was administered subcutaneously each day, beginning with the day of operation. This group was similarly divided into three groups of ten animals each. Dosage levels of 1,500 mg./Kg., 2,000 mg./Kg., and 2,500 mg./Kg. of histamine phosphate were injected intraperitoneally as in the previous determinations. The animals which succumbed at each dosage level were counted at the end of forty-eight hours and the number recorded.

Test for Completeness of Adrenalectomy.—In order to determine whether adrenalectomy was complete a random sample of six adrenalectomized, thymectomized rats, not used in the above determinations, was maintained on Rockland diet and normal saline for a period of twenty-four hours following surgery. At the end of this time, the saline was replaced with tap water and the mortality recorded during the next six days. The fact that all animals had succumbed at the end of this period was taken as evidence that adrenalectomy was complete. At the same time a control group of adrenalectomized, thymectomized rats was maintained on the same diet and saline for sixteen days following surgery, during which time no deaths occurred, providing ancillary evidence of completeness of adrenalectomy.

RESULTS

Table I shows the effect of dry-heat stress on both thymectomized and control rats. The LT_{50} for control rats of ninety-six minutes with 19/20 confidence limits of 87.8 to 106.2 minutes does not differ significantly according to significance of difference test (8) from the one hundred and five minute LT_{50} for the thymectomized group.

Tables II and III show the effect of reserpine-induced stress on control and thymectomized rats, respectively. The reserpine LD_{50} for thymectomized rats of 62.0 mg./Kg. falls outside the 19/20 confidence limits of control rats of 42.5 mg./Kg. but does not differ significantly from the LD_{50} of control rats according to significance of difference test.

Tables IV and V show the effect of histamine-induced stress on control and thymectomized rats, respectively. The histamine LD_{50} for thymectomized rats of 1,950 mg./Kg. falls within the 19/20 confidence limits of the histamine LD_{50} of control

[1] Serpasil Placebo Vehicle "C" was generously supplied by Ciba Pharmaceutical Products Co., Inc.

TABLE I.—EFFECT OF DRY-HEAT STRESS ON THYMECTOMIZED AND CONTROL RATS

Time in Minutes	No. Dead, Control	No. Dead, Thymexed	Cumulative Control	Cumulative Thymexed	% Resp. Control	% Resp. Thymexed
0– 85	0	0	0	0	0	0
85– 90	4	1	4	1	20	5
90– 95	5	5	9	6	45	30
95–100	5	4	14	10	70	50
100–105	2	3	16	13	80	65
105–110	3	1	19	14	95	70
110–115	0	1	19	15	95	75
115–120	0	1	19	16	95	80

Median lethal time (LT_{50})—96.0 minutes (control rats).
P 0.05 confidence limits—87.8 minutes–106.2 minutes.
LT_{50} for thymectomized rats—105.0 minutes.

TABLE II.—RESERPINE-INDUCED STRESS IN CONTROL RATS

Dose mg./Kg.	Mortality/Tested	% Response
25	3/10	30
50	5/10	50
75	8/10	80

LD_{50}—42.5 mg./Kg.
P 0.05—confidence limits (30.3 mg./Kg.–59.5 mg./Kg.).

TABLE III.—RESERPINE-INDUCED STRESS IN THYMECTOMIZED RATS

Dose mg./Kg.	Mortality/Tested	% Response
25	2/10	20
50	3/10	30
75	6/10	60

LD_{50}—62.0 mg./Kg.

TABLE IV.—HISTAMINE-INDUCED STRESS IN CONTROL RATS

Dose mg./Kg.	Mortality/Tested	% Response
800	2/10	20
1,200	5/10	50
1,600	6/10	60

LD_{50}—1320 mg./Kg.
P 0.05—confidence limits (713.5 mg./Kg–2,442 mg./Kg.).

TABLE V.—HISTAMINE-INDUCED STRESS IN THYMECTOMIZED RATS

Dose mg./Kg.	Mortality/Tested	% Response
800	1/10	10
1,200	4/29	13.8
1,600	9/29	31
2,000	11/19	57.9

LD_{50}—1,950 mg./Kg.

TABLE VI.—HISTAMINE INDUCED STRESS IN THYMECTOMIZED-ADRENALECTOMIZED RATS

Dose mg./Kg.	Mortality/Tested	% Response
400	3/9	33.3
700	4/9	44.4
1,000	8/10	80.0

LD_{50}—630 mg./Kg.

TABLE VII.—HISTAMINE-INDUCED STRESS IN THYMECTOMIZED-ADRENALECTOMIZED RATS MAINTAINED ON DOCA (0.25 MG./DAY)

Dose mg./Kg.	Mortality/Tested	% Response
1,500	3/10	30
2,000	6/10	60
2,500	10/10	100[a]

[a] 94.3% corrected value (Litchfield-Wilcoxon).
LD_{50}—1,800 mg./Kg.

rats of 1,320 mg./Kg. and does not differ significantly from the latter according to significance of difference test.

Table VI shows the effect of histamine-induced stress on thymectomized-adrenalectomized rats. The histamine LD_{50} for thymectomized-adrenalectomized rats of 630 mg./Kg. falls outside the 19/20 confidence limits of the histamine LD_{50} of intact, control rats of 1,320 mg./Kg., and differs significantly from the latter according to significance of difference test.

Table VII shows the effect of histamine-induced stress on thymectomized-adrenalectomized rats maintained on DOCA. The histamine LD_{50} of thymectomized-adrenalectomized rats maintained on DOCA, which was 1,800 mg./Kg., falls within the 19/20 confidence limits of the histamine LD_{50} of intact, control rats, which was 1,320 mg./Kg., and does not differ significantly from the latter according to significance of difference test.

DISCUSSION

Since there occurred no significant difference in stress resistance following thymectomy, it would appear that the thymus gland has little bearing on stress resistance. Moreover, it was shown that stress resistance can be restored from below normal to normal by the administration of adrenal cortical hormones to adrenalectomized animals without an attendant involution of the thymus gland. (This is true in the case of normal-thymus animals which have been thymectomized.) This would indicate that the process of involution of the thymus gland is not essential to the return of normal stress resistance. Additional work is planned to determine the resistance to stress of a group of rats with experimentally enlarged thymus glands. This should help to clarify the relationship between the thymus gland and stress resistance.

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An Investigation of the Blood Coagulating Principles from *Ceanothus Americanus**

By T. A. LYNCH†, T. S. MIYA, and C. JELLEFF CARR

Identification and evaluation of the blood coagulating principles from the root bark of *Ceanothus Americanus* was made. An acidic fraction of the drug was prepared by an ion exchange resin method. The acidic constituents identified by paper chromatographic techniques were four dicarboxylic and two phosphoric acids. All of these acids possessed a diphasic blood coagulating action as determined by an electrical conductivity method using the blood of calcium treated rats.

Ceanothus Americanus, used empirically in therapeutics for many years, has been investigated principally for vasodepressor (1–3) and hemodynamic activity (1, 2, 4–7). In order to clarify the views on the blood coagulant property, the identification of the active principles was made.

The purpose of this study was to separate and identify the acidic constituents of the plant in which the activity was found to reside, and to evaluate these acidic constituents for their blood coagulant property.

EXPERIMENTAL

Preparation of Plant Fractions.—(*a*) A hydroalcoholic extract of *Ceanothus Americanus* was prepared by macerating 100 Gm. of the powdered root bark in a percolator for two days with 500 ml. of 70% ethanol, previously warmed to 65°. After the extract was withdrawn, enough menstruum was passed through the percolator to make a total volume of 500 ml. (*b*) An acidic fraction was prepared using 35 Gm. of a strong Amberlite® ion exchange resin, IRA-400. The resin was lightly packed into a 2 x 38-cm. glass column. One hundred ml. of the hydroalcoholic extract was diluted to 300 ml. with 70% ethanol and passed through the column. Elution of the acids from the resin was effected by passing 300 ml. of 2% sodium hydroxide in 70% ethanol through the column. The effluent, containing the acidic constituents in an alkaline medium, was then passed through a strong cationic Amberlite exchange resin, IR-120, to remove the sodium ion. Three hundred ml. of effluent, containing the acidic constituents in alcoholic solution and appearing as an amber colored solution was collected, representing 20 Gm. of the root bark. (*c*) Three hundred ml. of the effluent obtained by passing the hydroalcoholic extract through the IRA-400 resin column constituted the neutral-basic fraction.

Paper Chromatography.—Chromatographic chambers consisted of an airtight battery jar (13 L.) in which a 4-L. beaker containing 50 ml. of solvent was placed. Samples (3–25λ) were spotted at points 3.5 cm. apart on a line 5 cm. from the bottom of a 25 x 25-cm. Whatman No. 1 filter paper. The diameter of the spot was kept constant. The ends of the paper were fastened to form a cylinder which was then placed in the 50 ml. of solvent and allowed to ascend for four and one half hours. After drying overnight at room temperature, the paper was developed by spraying with bromocresol blue (40 mg./100 ml. 95% ethanol).

Extensive preliminary chromatographic studies of certain known organic acids implicated oxalic, tartaric, citric, malic, malonic, succinic, citraconic, orthophosphoric, and pyrophosphoric acids as possible constituents of the acidic fraction. Comparative ascending one-dimensional paper chromatograms of the acidic fraction and certain known acids which were prepared using two different solvent systems are shown in Figs. 1 and 2.

In order to establish the identity of the above acids in the acidic fraction, ascending two-dimensional paper chromatography was employed. Paper chromatography of the acidic fraction (25λ) using *n*-butanol-acetic acid-water (4:1:1) and water-saturated phenol produced several acidic spots. These spots were eluted from the paper with distilled water. One-half of the total eluant was spotted separately and the other half spotted together with 5λ of a known 1% acid at a point 5 cm. from adjacent edges of a 25 cm. square, Whatman No. 1 filter paper. The paper cylinder was first placed into the water-saturated phenol, dried, and then placed in the *n*-butanol-acetic acid-water (4:1:1) solvent to run in the other dimension. One distinct spot occurring on each pair of chromatograms constituted a positive test for the acid.

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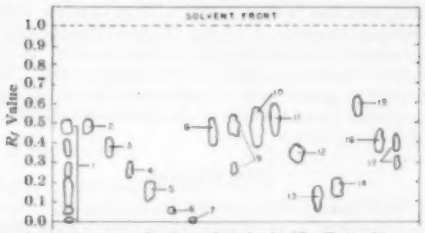
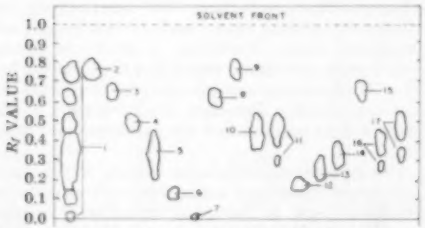


1	Acidic fraction	7	Pyrophosphoric	13	Tartaric
2	Succinic	8	Glycolic	14	Citric
3	Malonic	9	Citraconic	15	Lactic
4	Malic	10	Maleic	16	α-Ketoglutaric
5	Oxalic	11	Itaconic	17	*cis*-Aconitic
6	Orthophosphoric	12	Pyruvic		

Fig. 1.—Drawing of comparative ascending one-dimensional paper chromatograms of the acidic fraction (25λ) and known 1% acids (3λ) with water-saturated phenol.



1	Acidic fraction	7	Pyrophosphoric	13	Tartaric
2	Succinic	8	Glycolic	14	Citric
3	Malonic	9	Citraconic	15	Lactic
4	Malic	10	Maleic	16	α-Ketoglutaric
5	Oxalic	11	Itaconic	17	*cis*-Aconitic
6	Orthophosphoric	12	Pyruvic		

Fig. 2.—Drawing of comparative ascending one-dimensional paper chromatograms of the acidic fraction (25λ) and known 1% acids (3λ) with *n*-butanol-acetic acid-water (4:1:1).

Oxalic, malonic, malic, succinic, orthophosphoric, and pyrophosphoric acids were identified by this method. The R_f values obtained by the two-dimensional chromatograms corresponded closely to those of the one-dimensional chromatograms shown in Figs. 1 and 2.

A confirmatory identification test for phosphates was carred out by a color reaction. One-dimensional and two-dimensional chromatograms of the sodium salts of ortho-, meta-, and pyrophosphate, and the acidic fraction were prepared. Development of the chromatograms was carried out by a procedure described by Cramer (8). Blue spots appeared on the chromatograms at the sites of the ortho-, meta-, and pyrophosphate, and at two positions on the chromatograms prepared from the acidic fraction. These corresponded to the R_f values as previously determined for the ortho- and pyrophosphate. A phosphate test was also conducted which differentiates the pyro- from the ortho- and the metaphosphate. The method used was that of Ando, *et al.* (9), employing paper chromatographic technique. The test indicated the presence of orthophosphate and traces of pyrophosphate.

Chromatographic Estimation of the Acids of Ceanothus Americanus.—Quantitative assay of the acids identified in *Ceanothus Americanus* was effected by the paper chromatographic method of Beerstecher (10) using *n*-butanol-acetic acid-water (4:1:1). The data in Table I show that succinic acid was present in the highest concentration, followed in order by oxalic, malonic, malic, orthophosphoric, and pyrophosphoric acid.

Blood Coagulation, General Procedure.—The method devised by Richardson and Bishop (11) which measures the electrical conductivity of blood as a means of determining blood clotting time was found to be most suitable. The blood coagulometric apparatus was constructed according to the specifications of its authors, with one exception. The oscillation rate of the platform per second was 6 instead of 10.

One ml. of blood was withdrawn from an etherized Sprague-Dawley rat by cardiac puncture with a mineral oil-coated, 23-gauge needle and a 1 ml. tuberculin syringe. A stopwatch was started the instant the blood was withdrawn. The needle was removed and 0.5 ml. of blood was gently discharged into the blood chamber coated with mineral oil.

Preliminary Blood Clotting Time Determinations.—A number of preliminary tests indicated that the neutral-basic fraction given orally or added directly to blood was without clotting activity. The hydroalcoholic and acidic fractions significantly reduced blood clotting time by about 25%. No apparent difference was observed in the clotting activity of these fractions when administered orally or added directly to blood. The latter findings are similar to those of Bishop and Richardson (7) who employed dogs.

An important source of variation in blood clotting time in rats was observed during the course of the investigation. Certain amounts of oxalate added to the blood of mature rats significantly diminished clotting time, while the same amount of oxalate added to the blood of young rats (100–140 Gm.) prolonged clotting time. It was noted that the normal blood clotting time of the young rats was approximately the same as that produced by the addition of sodium oxalate to the blood of mature rats (225–235 Gm.).

It was found that by orally administering the young rats calcium gluconate, the blood clotting time after fifteen minutes was approximately the same as that of the mature rats. Oxalate added to the blood of these calcium treated rats significantly reduced clotting time. Very little effect was noted by calcium administration to mature rats. The data in Fig. 3 graphically illustrates the above phenomenon.

Blood Clotting Activity of the Acids Identified in Ceanothus Americanus.—The acids identified in *Ceanothus Americanus* were tested as the sodium

TABLE I.—CONTENT OF ACIDS IN THE ACIDIC FRACTION OF *Ceanothus Americanus* AS DETERMINED BY PAPER CHROMATOGRAPHIC ASSAY

Acid	%
Succinic	0.150
Oxalic	0.130
Malonic	0.051
Malic	0.049
Orthophosphoric	0.038
Pyrophosphoric	0.0005

salts at different concentrations for clotting activity using rat's blood. Since the blood clotting time in all groups of rats was apparently stabilized by 5% calcium gluconate, 1 ml./75 Gm. of rat weight was orally administered throughout the tests for both control and drug groups. Clotting time determinations were made by placing known amounts of the sodium salts of the acids (0.1 ml.) in the coagulometer tube immediately prior to the addition of the blood. Control and test runs were made alternately. The amount of salt to which the blood was added was expressed in terms of the weight of the free acid. Ten to fifty rats were used at each blood level of the salt.

The results shown in Table II show the mean per cent change of each acid salt at different blood concentration. All of the salts exerted a diphasic blood coagulant effect. Oxalate was the most active coagulant, followed by orthophosphate, malonate, malate, and pyrophosphate which were about equally effective. Succinate was the least active. The oxalate and malonate exhibited the widest range of activity; however, the malonate curve was quite erratic.

Blood Coagulating Activity of Combinations of the Acids of Ceanothus Americanus—Various combinations of the acids were tested as the sodium salts for blood coagulating activity. The data in Table III indicate that the combined effect of the acids is merely additive.

Blood Coagulating Activity of Itaconic Acid.—Itaconic acid, an unsaturated, hydroxy dicarboxylic acid was also tested as the sodium salt for blood clotting activity. A 10 mg. % blood concentration diminished the clotting time of rat blood by 16.4% below 5 controls (significant at the 5% level). This finding suggests that unsaturation does not appreciably alter the effect of the dicarboxylic acids on blood coagulation.

DISCUSSION

The findings of Bishop and Richardson (6, 7) have demonstrated that certain amounts of a hydroalcoholic extract of *Ceanothus Americanus* administered orally or added directly to the blood of dogs exerted a coagulant effect which was attributed to removal of small amounts of blood calcium. Larger amounts of the extract prolonged clotting time. In this study, a similar coagulant effect of the drug was observed in rats, the maximum diminution in clotting time being approximately 25%.

The lack of uniformity in blood clotting time observed in different weight rats could be attributed to inherent differences in the level of blood calcium and calcium-complexing agents such as dicarboxylic and phosphoric acids. Variations in intestinal absorption of these substances might be a contributing factor. These assumptions are made in view of the fact that oral calcium gluconate administration to different weight rats reduced the variations in clotting time.

The diphasic hemodynamic nature of the dicarboxylic and phosphoric acids, the active coagulating principles found in *Ceanothus Americanus*, suggests

TABLE II.—BLOOD CLOTTING ACTIVITY OF THE SODIUM SALTS OF THE ACIDS OF *Ceanothus Americanus* ADDED TO THE BLOOD OF CALCIUM GLUCONATE[a] TREATED RATS

Salt[b]	Blood[c] Concn., mg. %	No. of Animals[d]	Clotting Time, Mean % Change and S. E.[e]	Significance Level[f]
Oxalate	5	10	−11.0 ± 4.9	0.01
	10	12	−23.3 ± 5.6	0.01
	20	10	−16.3 ± 3.4	0.01
	30	10	+10.1 ± 6.9	0.30
Malonate	1.8	10	+10.1 ± 14.5	Not significant
	5	10	−20.6 ± 9.0	0.10
	10	30	−17.7 ± 5.8	0.01
	12.3	10	+3.9 ± 1.6	0.10
	18.3	10	−20.6 ± 4.7	0.05
	20	20	−14.6 ± 7.7	0.01
	30	10	−3.4 ± 3.8	0.50
	40	10	+0.9 ± 12.6	Not significant
Malate	5	10	−13.2 ± 7.0	0.20
	10	10	−20.3 ± 15.6	0.30
	15	10	−14.8 ± 4.2	0.05
	20	10	+7.8 ± 6.4	0.30
	40	10	+3.8 ± 4.0	0.40
Succinate	5	10	−6.0 ± 7.7	0.50
	10	50	−15.0 ± 8.5	0.10
	15	10	−6.9 ± 10.8	Not significant
Orthophosphate	5	10	−11.9 ± 3.4	0.05
	10	10	−19.3 ± 8.2	0.10
	20	10	+10.0 ± 4.0	0.10
Pyrophosphate	5	10	−17.3 ± 6.2	0.05
	10	10	+10.4 ± 6.0	0.20

[a] Five per cent calcium gluconate (13.3 ml./Kg.) given orally 15 minutes before taking blood sample.
[b] Sodium salt of acid was used.
[c] One-half ml. of blood added to 0.1 ml. of salt solution.
[d] One-half of the number were used as controls; clotting time 90–120 seconds.
[e] Standard error of the mean per cent change.
[f] Student's *t* test.

TABLE III.—BLOOD CLOTTING ACTIVITY OF DIFFERENT MIXTURES OF THE SODIUM SALTS OF THE ACIDS OF *Ceanothus Americanus* ADDED TO THE BLOOD OF CALCIUM GLUCONATE[a] TREATED RATS

Sodium Salts	Mixture Ratio	Total[b] Blood Concn., mg. %	No. of Animals[c]	Clotting Time, Mean % Change and S. E.[d]	Significance Level[e]
Oxalate:Malonate	1:1	10	10	−13.9 ± 3.7	0.05
Malonate:Succinate	1:1	10	10	−21.2 ± 6.3	0.05
		20	20	−10.1 ± 13.5	0.50
Oxalate:Malate	1:1	10	10	−19.5 ± 3.4	0.01
		20	10	−10.9 ± 2.7	0.10
Oxalate:Orthophosphate	1:1	10	10	−17.3 ± 4.3	0.01
		20	10	−10.4 ± 6.0	0.20
Oxalate:Pyrophosphate	2:1	7.5	10	−17.0 ± 2.9	0.01
		15	10	+2.6 ± 4.5	Not significant
All acid salts	[f]	5	10	−20.0 ± 3.3	0.01
		10	10	−8.8 ± 2.3	0.05
		15	10	0.0 ± 4.7	...

[a] Five per cent calcium gluconate (13.3 ml./Kg.) given orally 15 minutes before taking blood sample.
[b] One-half ml. of blood added to 0.1 ml. of salt solution.
[c] One-half of the number were used as controls—clotting time 90–120 seconds.
[d] Standard error of the mean per cent change.
[e] Student's *t* test.
[f] An equal amount of the 6 salts were used.

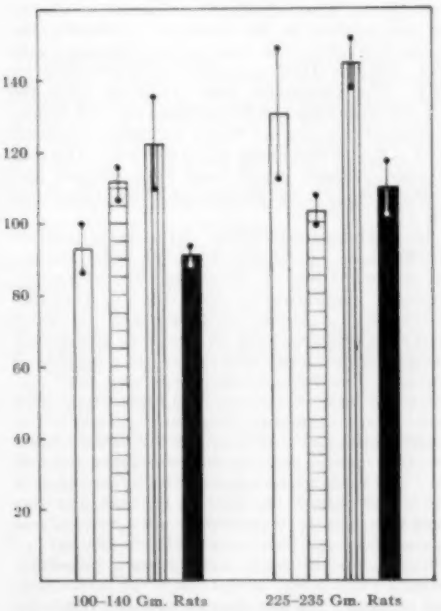


Fig. 3.—Clotting response of blood from different weight rats after various treatments. □ Normal; ▤ Na oxalate (10 mg. %) added to blood; ▥ 500–750 mg./Kg. Ca gluconate, orally, 15 minutes prior to coagulation test; ■ Na oxalate (10 mg. %) added to blood of Ca gluconate treated rats. ⊥ Standard error. Three to seven rats employed for each test.

that their mode of action is similar. The additive coagulant effect of mixtures of these acids supports this view. The apparent differences in molar effectiveness and range of activity of these coagulants may be due to the dissimilarity in their ionic charge and/or ability to complex blood calcium. The blood coagulant effect observed using itaconic acid suggests that chemical unsaturation does not alter the clotting activity of certain dicarboxylic acids.

Oxalate appears to be the most suitable coagulant on the basis of its greater range of activity and uniform effectiveness.

CONCLUSIONS

The separation and identification of the acidic constituents from the hydroalcoholic extract of the powdered root bark of *Ceanothus Americanus* is presented. These substances consist of 4 dicarboxylic and 2 phosphoric acids, all of which possess blood coagulating activity.

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The Flavonoid Constituents of *Amphiachyris dracunculoides* (DC) Nutt.*

By HOWARD J. LEVIN† and LOYD E. HARRIS

Utilizing a cation exchange resin, a crude flavonoid mixture was isolated from *Amphiachyris dracunculoides*. The flavonoid mixture was separated into four different compounds by paper chromatography and eluted. After acid hydrolysis, the flavonoid aglycons and the sugar moieties were chromatographed. Ultraviolet absorption spectral data were determined. Color reactions of the flavonoid compounds in visible and ultraviolet light were recorded. From these data it is indicated that two of the compounds were flavone aglycons, one, a flavonol-3-glucoside, and the other, a flavonol bioside containing glucose and arabinose.

Amphiachyris dracunculoides (DC) Nutt., Compositae, known by the common names of Broom Weed and August Flowers (1), is an annual herb, native to the south central portion of the United States. The use of this plant as a home remedy for coughs and colds has been established for many years. An infusion is prepared from the dried branching portion of the plant which includes the flowers and leaves. The liquid obtained in this manner is sometimes thickened to a syrupy consistency by the addition of a sufficient amount of sugar. A type of medicated cough drop has also been prepared.

Two phytochemical investigations of the plant had been reported previously (1, 2). In 1933, Harris and Griffith (1) reported the presence of a glycoside in an ethanolic extract of the plant. The characteristics of this substance, however, were not determined. Later, Glasser, *et al.* (2), reported an exhaustive investigation of the volatile oil present in the plant.

The empiric use of an infusion of *Amphiachyris dracunculoides* suggested that any reported biological action may be due to principles present in the water-soluble portion of the plant. The presence of flavonoid compounds in an aqueous extract was established by the writers. The current use of such compounds in the therapy of the common cold suggested a further investigation of this group of constituents.

EXPERIMENTAL

The samples of *Amphiachyris dracunculoides* used in this investigation were obtained from northwest Greer County, Oklahoma, and collected during August of 1956 and 1957. The parts of the plant used were the upper stems, leaves, and flowers; they were air dried at room temperature and then reduced to a number 20 powder.

Steam Distillation and Water Extraction.—A muslin sack containing a 1,500-Gm. portion of the comminuted plant was placed in a Lloyd extractor and directly steam distilled until no more volatile oil was evident in the distillate. Following the steam distillation, the material was extracted with 10-L. portions of boiling distilled water for two hours each. The extractions were continued until the Mg-HCl reduction test for the presence of flavonoid compounds (3) was no longer positive. A total of 50 L. of a brown liquid was collected. This was strained through muslin and kept without any preservative at a temperature of 5° until ready for use.

Ion Exchange Adsorption.—In order to separate the flavonoid constituents from the water extract, the method of Wender (4) was followed with modifications. Approximately 600 Gm. of Amberlite IRC-50(H),[1] a cation exchange resin in which activity is principally due to carboxylic acid groups in the hydrogen form, were reduced to a number 40 powder. Two Pyrex columns were used, each constricted at its lower end and each fitted with a stopcock. One column was 114 cm. in height and had an inner diameter of 4.50 cm., and the other column was 125 cm. high and had an inner diameter of 2.70 cm. Each column was packed with a 5-cm. layer of Pyrex wool, followed by 5 cm. of sea sand, and then filled with a slurry of the resin to a height of 97 cm. in the former and 105 cm. in the latter column.

The 50 L. of water extract were percolated through the columns at the rate of 10 L./hour/sq. dm. of resin. The solution that passed through the columns had about the same outward appearance as on entry, but it now gave a negative Mg-HCl reduction test (3). During the percolation, the resin gradually acquired a yellow tint. The extract was followed on the columns with a wash of distilled water until the washings were colorless. The columns were then washed with 95% ethanol in order to elute the flavonoid compounds. A total of 8 L. of orange-brown colored hydro-alcoholic solution was obtained.

The hydro-alcoholic solution was concentrated to 700 ml. This concentrate, now highly aqueous, contained some finely divided yellow solid and some sticky, dark-brown solid material. This mixture was diluted to 15 L. with distilled water, and to this was

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[1] Rohm and Haas Company, Philadelphia, Pa.

added 25 Gm. of solid potassium acetate in order to form the potassium salts of the flavonoid compounds. Some finely divided yellow-brown solid material appeared suspended, and a few drops of 10% KOH were added to complete solution. The pH of the resulting solution was approximately neutral. The solution was percolated through the regenerated columns described above and treated in the same manner. Two liters of yellow colored hydroalcoholic solution were obtained.

This solution was concentrated to about 100 ml. by distillation under reduced pressure. It now contained some yellow and brown solids. The mixture was diluted to 1 L. with distilled water and extracted with *n*-butanol in a liquid-liquid extractor until the water mixture no longer gave a positive Mg-HCl test (3). The yellow *n*-butanol solution was concentrated to dryness by distillation under reduced pressure and the crude flavonoid mixture weighed 4.85 Gm. A melting point determination was attempted but proved unsatisfactory since the material softened over a considerable temperature range.

Paper Chromatography.—The descending method was used. Strips of Whatman No. 1 filter paper, having the dimensions 3.8 by 60.7 cm., were cut and about 200–400 μg. of the crude flavonoid mixture were applied to the paper, by spotting from 0.01–0.02 ml. of a 2% solution in ethanol at the 10 cm. origin line. After development, the compounds were detected by their varying shades of yellow and orange under ultraviolet light.

Investigations using eight different solvent systems showed that the best separation of the components of the mixture was achieved by using a solvent system composed of acetic acid-water, 3:2 by volume. The average of twelve chromatographic determinations revealed four components designated Flavonoid A (R_f = 0.23 – 0.29), Flavonoid B (R_f = 0.42), Flavonoid C (R_f = 0.56), and Flavonoid D (R_f = 0.72).

Isolation of Flavonoids.—The same paper chromatographic procedure as described above was used with 0.06 ml. of a 2% ethanolic solution of the crude flavonoid mixture spotted on 110 strips. After development with the 60% acetic acid and air drying, the strips were each cut into four segments, each segment containing one spot corresponding to one of the four flavonoid constituents. The segments were separated into four groups as designated, reduced to a number 10 powder, and then extracted with ethanol for twenty-four hours in a Soxhlet apparatus. The resulting four fractions were concentrated by distillation under reduced pressure and then dried to constant weight in a vacuum oven at 60° over P_2O_5. The amounts recovered were 37.2 mg. of Flavonoid A, 10.5 mg. of Flavonoid B, 14.0 mg. of Flavonoid C, and 68.4 mg. of Flavonoid D.

Hydrolysis of Flavonoids.—One-half of the obtained amounts of each of the flavonoids were hydrolyzed with HCl. A suspension of each flavonoid and 10 ml. of distilled water containing 1 ml. of concentrated HCl were heated on a steam bath overnight in a 25-ml. round-bottom flask connected with a reflux condenser. After cooling to room temperature, the solid materials were filtered and dissolved in ethanol-ether, 1:1 by volume. The alcohol was removed by washing with water and the ether solutions dried with anhydrous Na_2SO_4. The ether solutions were evaporated to dryness and the residues designated as Aglycons A, B, C, and D.

Each original aqueous filtrate from the hydrolyses was extracted with five 10-ml. portions of ether and the HCl removed by shaking the solutions with Ag_2CO_3 (until the pH was neutral) and filtering through a layer of charcoal (5). The colorless filtrates were concentrated, under reduced pressure, to volumes of 3 ml. each.

Identification of Sugars.—The filtrates from the hydrolysis of the flavonoid constituents were examined with paper chromatography. The upper layer of the 2-phase solvent system of a mixture of *n*-butanol–acetic acid–water, 4:1:5 by volume, was used to develop the chromatograms. The chromatograms, after development for about seventeen hours, were sprayed with a solution of aniline hydrogen phthalate in water-saturated *n*-butanol (6) and then dried at 100° for five minutes. Known sugars were used for comparisons. The paper chromatograms indicated that Flavonoids A and C contained no sugar, Flavonoid B contained glucose, and Flavonoid D contained glucose and arabinose.

R_f Values of Flavonoids and Aglycons.—Two per cent solutions of the hydrolyzed and unhydrolyzed flavonoids in ethanol were subjected to paper chromatography by use of the descending technique. Three solvent systems were used and the R_f values determined. The results are shown in Table I.

These results shown by paper chromatography in Table I verify the conclusions that Flavonoids A and C are aglycons and Flavonoids B and D are glycosides, but the R_f values do not coincide with values of known compounds surveyed in the literature.

Spectral Data of the Flavonoid Compounds.—Ultraviolet absorption spectra of Flavonoids A, B, C, and D were measured in 95% ethanol. The concentrations of the solutions were 0.0025%. The instrument used was the Beckman Model DU Spectrophotometer, Serial No. 61828, with the hydrogen discharge bulb as the light source, and values were plotted from 245 to 385 mμ. The silica cells used were 1.001 cm. wide.

The data obtained from the absorption spectra are reported in tabular form in Table II. The $E(1\%,1\text{ cm.})$ values are given in respective order of the positions of maximum absorbance.

From the spectral data obtained and its comparison with data surveyed in the literature by the author, no exact comparisons were encountered. However, the positions of maximum absorbance

TABLE I.—R_f VALUES OF FLAVONOIDS AND AGLYCONS

	n-Butyl Alcohol-Acetic Acid-Water, 4:1:5, by Vol.	Water-Saturated Ethyl Acetate	Acetic Acid-Water, 3:2, by Vol.
Flavonoid A	0.77	0.93	0.29
Flavonoid B	0.53	0.02	0.42
Flavonoid C	0.71	0.93	0.56
Flavonoid D	0.79	0.02	0.72
Aglycon A	0.77	0.93	0.29
Aglycon B	0.73	0.93	0.27
Aglycon C	0.71	0.92	0.56
Aglycon D	0.87	0.92	0.66

TABLE II.—SPECTRAL DATA OF FLAVONOID COMPOUNDS

Compound	Positions of Maximum Absorbance (mμ)	E(1%,1 cm.)
Flavonoid A	258; 321	198; 93
Flavonoid B	253; 325; 343	512; 279; 313
Flavonoid C	273; 332	388; 281
Flavonoid D	268; 345	293; 130

shown in Table II are approximately within the known regions of maximum absorption for flavones and flavonols, *i. e.*, 240–260 mμ and 330–375 mμ (7). From observations of the positions of the longer wavelength maxima, that is, on the basis of comparison with spectral data of known flavonoids (7), Flavonoids A and C were flavones, and Flavonoids B and D were flavonols.

Color Reactions of the Flavonoid Compounds.—The paper chromatograms of Flavonoids A, B, C, and D were examined under visible and ultraviolet light both untreated and after spraying with chromogenic reagents. The reagents used were 1% solutions of sodium carbonate, aluminum chloride, neutral lead acetate, and basic lead acetate in the manner of Casteel and Wender (8). The colors produced by the chromogenic sprays for the four isolated flavonoid compounds are shown in Table III.

TABLE III.—FLAVONOID COMPOUNDS. COLORS PRODUCED BY CHROMOGENIC SPRAYS[a]

Flavonoid	Untreated		1% Aqueous Na_2CO_3		1% Alcoholic $AlCl_3$		1% Aqueous PbAc		1% Aqueous PbOAc	
	Vis.	U. V.	Vis.	U. V.	Vis.	U. V.	Vis.	U. V.	Vis.	U. V.
A	C	BY	C	I	C	Y	C	Y	T	Y
B	C	OBr	Y	Y	Y	Y	Y	O	Y	O
C	C	YBr	Y	YG	C	YG	PY	I	T	I
D	C	Y	C	I	YBr	YG	YG	O	YBr	OBr

[a] Colors symbolized as follows: C, colorless; B, bright, P, pale; Y, yellow; O, orange; Br, brown; I, ivory; G, green; T, tan

DISCUSSION

Judging from the evidence obtained from paper chromatography, ultraviolet spectral data, and color reactions, Flavonoid A was a flavone aglycon. The absence of a visible color with aluminum chloride indicated the absence of a 3- or 5-hydroxyl group and the absence of any adjacent pair of hydroxyl groups in the molecule.

Flavonoid B was indicated to be a flavonol-3-glucoside. This conclusion was obtained from the orange-brown color observed under ultraviolet light when untreated, and the yellow color when treated with sodium carbonate solution. The yellow color obtained with aluminum chloride solution also indicated that either a hydroxyl group was present at position 5, or that an adjacent pair of hydroxyl groups were present elsewhere in the molecule. Spectral data and R_f values before and after hydrolysis substantiated this conclusion.

Flavonoid C appeared to be a flavone aglycon as shown by spectral data and paper chromatography. This was substantiated by the yellow-brown color observed on the untreated chromatogram under ultraviolet light, by the yellow color when treated with sodium carbonate solution, and by the absence of color when treated with aluminum chloride. These reactions also suggested the absence of a 5-hydroxyl group and the absence of any adjacent pair of phenolic hydroxyl groups.

Flavonoid D was indicated to be a flavonol glycoside containing arabinose and glucose as previously determined by hydrolysis, spectral data, and R_f values before and after hydrolysis. It was observed from Table III that the glycosidic linkage was not at position 3. This was apparent from observation of the untreated chromatogram under ultraviolet light. This was substantiated by the colors obtained with aluminum chloride and neutral lead acetate.

SUMMARY AND CONCLUSIONS

1. Four different flavonoid compounds have been isolated using the techniques of ion exchange adsorption and paper chromatography. Two of these compounds have been hydrolyzed and shown to be glycosides by paper chromatography. R_f values of the glycosides and aglycons were determined in three solvent systems.

2. The sugar moieties of the flavonoid glycosides have been determined. One was shown to contain glucose and the other, glucose and arabinose.

3. Ultraviolet adsorption characteristics of the four flavonoid compounds have been recorded in tabular form.

4. Color reactions of the four flavonoid compounds in visible and ultraviolet light have been determined and are presented in tabular form.

5. The experimental evidence favors the conclusion that two of the flavonoid compounds are flavone aglycons; one was indicated to be a flavonol-3-glucoside, while the other is apparently a flavonol bioside containing glucose and arabinose.

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The Application of Ultrasonic Energy to the Extraction of Belladonna Leaf U. S. P.*

By PAUL E. WRAY and LAVERNE D. SMALL

The ammoniated ether-alcohol maceration mixture of the powdered belladonna leaf U. S. P. XV was first subjected to a twenty-minute treatment with ultrasonic energy, having a vibrational frequency of 500 kilocycles per second. This treatment made it possible to eliminate the overnight maceration period and to obtain complete maceration of the drug in three hours in a Soxhlet extractor. A more efficient utilization of these ultrasonic vibrations in a specially designed treatment vessel resulted in an additional saving of time. A chromatographic analysis of the material extracted, with the aid of ultrasonic energy, failed to show any decomposition products of the alkaloids. The dried marc from the ultrasonic treated maceration samples showed some particle size increase, but the dried marc from the submerged ultrasonic extraction samples showed some particle size decrease. The results obtained indicate that some extraction of the alkaloids of belladonna leaf U. S. P. is facilitated by the application of ultrasonic energy.

THE ACCELERATION of partition extraction by the application of ultrasonic energy was demonstrated in 1950 by Haul, Rust, and Lutzow (1). They noted that the use of ultrasonic energy considerably increased the amount of phenanthrene extracted from methyl alcohol by a petroleum fraction.

In 1952 Lepeschkin and Goldman (2) studied the effect of ultrasonic vibrations on plant cells. The cells, when subjected to ultrasonic vibrations, exhibited increased permeability to several solvents.

Specht (3), in 1952, and Schroder (4), a year later, extracted the bitter substances from hops with the aid of ultrasonic energy. Both noted a more efficient extraction of the hops resulting in a saving of time and material. Thompson and Sutherland (5), in 1955, reported the extraction of oil from crushed peanuts by hexane in the presence of a 400-kilocycle ultrasonic field. Then in 1956, Head, Beal, and Lauter (6) confirmed some of the evidence which had already been obtained in this investigation. They accomplished the extraction of *Cinchona succirubra*, notorious for its difficulty of extraction, by the use of ultrasonic energy. The results of these investigations in addition to other known effects of ultrasonic energy, such as agitation, increase of temperature, and a possible reduction of particle size seemed to warrant a study of the application of ultrasonic energy to drug extraction. It was decided to determine the usefulness of ultrasonic vibrations in the extraction of belladonna leaf U. S. P. XV (7) and to ascertain, if possible, whether any lessening of the maceration or extraction periods were largely due to a reduction of the particle size of the drug.

A chromatographic analysis of the alkaloids extracted with the aid of ultrasonic treatment was planned. It was thought that perhaps the heat and vibrational effects of the ultrasonic energy might decompose the alkaloids. The circular paper chromatographic procedure of Romeike (8) was used. This procedure was reported to give separation of atropine, scopolamine, tropine, and scopoline. Thus, hydrolysis products of atropine, such as tropine and tropic acid, could be detected by the Romeike method.

Belladonna leaf U. S. P. XV was chosen for this study for two reasons. First, the alkaloids of *Atropa belladonna* L. are of major pharmaceutical interest since they are widely used medicinally. Secondly, a reliable, official method of extraction and assay is available in the U. S. P. XV (7). The latter reason is of importance because it would enable the investigator to compare accurately the influence of ultrasonic treatment on the extraction of alkaloids with recognized methods.

The belladonna leaf was reduced to the proper state of subdivision, extracted, and assayed by the U. S. P. XV methods (7). The minimum time required to insure complete extraction of the drug following the official maceration and Soxhlet extraction procedure was then ascertained. Knowledge of this minimum time was necessary, since the excessive Soxhlet extraction of the sample would make accurate comparison of ultrasonic and official maceration procedures impossible. The duration of ultrasonic macera-

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tion treatment necessary to replace the U. S. P. overnight maceration time was then determined.

Newton and Kissel (9), in 1953, reported that the use of a submerged treatment vessel gave greater transmission of ultrasonic energy. A special treatment vessel was designed so that it could be completely submerged and would permit much of the energy from the crystal to pass through the sample without any of this energy being reflected at the liquid-air interface (Fig. 1 b). This more efficient utilization of the ultrasonic energy permitted maceration and extraction of the drug in a single, relatively short operation.

EXPERIMENTAL

Materials.—The belladonna leaf U. S. P. XV was ground to conform to the specifications for a moderately course powder as described in U. S. P. XV (10). U. S. P. grade solvents and reagents were used.

Equipment.—A Wiley Intermediate Model Laboratory Mill was used to grind the drug. The ground drug was mixed in a Patterson-Kelley Twin-Shell Blender, Model No. LB-1015. The sieves used were of the U. S. Standard Sieve Series made to the American Society of Testing Materials specifications. A Cenco-Meinzer Sieve Shaker was also used.

The ultrasonic generator used in these experiments was constructed by William S. Proudfit and described in his thesis (11). The ultrasonic generator operates at a frequency of 500 kc. It is composed of a radio frequency oscillator from which power is fed at high voltage to a quartz crystal immersed in an oil bath. During operation of the oscillator, the oil is circulated by means of a pump and is cooled by passing the oil through a coil of copper tubing that is placed in ice water. In these experiments, the generator was operated at 2,400 volts plate voltage and 133 ma. plate current applied to the type 4-250-A Eimac power tube. The output at the crystal face was 295 ma. The diameter of the crystal face is 3.87 cm., therefore, the energy at the crystal face is, 25.87 ma., per cm^2. Attempts were made to measure the potential difference across the faces of the crystal, but an accurate measurement was impossible. Detuning of the generator resulted when a vacuum tube volt meter was attached across the crystal faces. The detuning gave rise to considerable heating in the generator.

The material to be treated was placed in the treatment vessel, attached to an adjustable support, and lowered into the oil bath. Two types of ultrasonic treatment vessels were used.

The vessel used for ultrasonic maceration was a 50-ml. treatment vessel obtained from Ultrasonic Engineering Company (Fig. 1 a). It was a stainless steel cylinder with a thin steel (0.001 inch) diaphragm in the bottom. The sides of the vessel were extended with a 6-inch piece of Pyrex tubing of correct diameter attached by cellophane tape. This was added to prevent loss of material during the ultrasonic treatment due to the violent agitation. The thin diaphragm was held in place by a retaining ring and leakage was prevented by using neoprene gaskets. This vessel was operated on or near the surface of the oil in the oil bath.

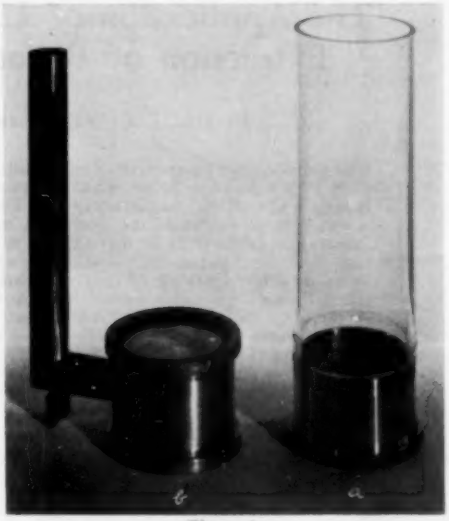

Figure 1.

The extraction vessel was of the submersion type (Fig. 1 b). It was made of brass with 0.001 inch thick diaphragms at top and bottom which were held in place with retaining rings, and neoprene gaskets were used to prevent leakage. Filling was accomplished through the side tube. The volume of the vessel was 100 ml. The total volume depended on the amount of liquid in the side tube and was approximately 110 ml.

Procedure.—The belladonna leaf, as obtained from the commercial supplier, was in a coarse state of subdivision. The drug was first thoroughly mixed in the Twin-Shell Blender for thirty minutes, then reduced to a moderately coarse powder in the Wiley Mill.

Particle Size Distribution.—The size distribution was obtained by using a series of sieves, numbers 60, 80, 100, 140, 170, 200, and 230. The sieves, containing 100 Gm. of ground drug in the No. 60 sieve, were shaken in the sieve shaker, with a medium sharp impact, for five minutes. The amount retained by each sieve was weighed on a Class A, torsion balance. A total of three determinations was made. The average weights of drug in the sieves are shown in Table I for the untreated drug. The separate portions of the drug were recombined and mixed in the Twin-Shell Blender. Six 10-Gm. samples of the drug were extracted and assayed by the U. S. P. XV method. The alkaloid content was found to be 0.40%.

Extraction Time Following Overnight Maceration.—An experiment was designed to determine the minimum time necessary to extract the alkaloids by Soxhlet extraction following the official overnight maceration period. It was found that the full three-hour extraction period was necessary to remove the alkaloids in amounts equivalent to the U. S. P. XV method (Fig. 2).

TABLE I.—PARTICLE SIZE ANALYSIS OF POWDERED DRUG, PER CENT RETAINED BY SIEVE

Drug	60	80	100	140	170	200	230	>230	Total
Untreated	43.34	27.22	7.35	7.95	3.44	2.78	1.71	5.31	99.10
Ultrasonic maceration	45.40	27.05	8.50	8.90	2.72	2.08	1.20	2.75	98.60
Ultrasonic extraction	35.00	28.80	9.40	9.10	5.00	4.00	2.05	4.80	98.15

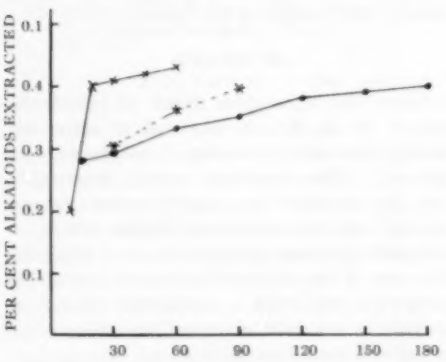

Fig. 2.—Extraction time (in minutes). • U. S. P. method. x Ultrasonic method (5 Gm. Sample). * Ultrasonic method (10 Gm Sample). Comparison of U. S. P. and ultrasonic extraction methods.

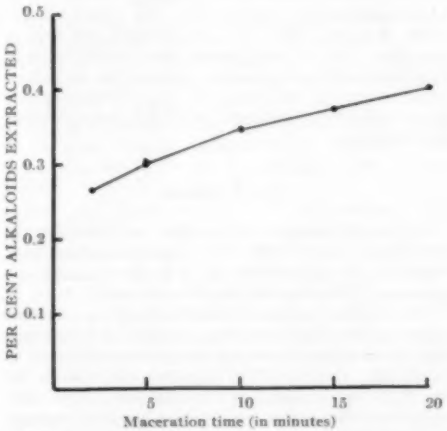

Fig. 3.—0.4% represents 100% of alkaloids extracted by U. S. P. XV method. Effect of ultrasonic maceration time on alkaloid extraction

Ultrasonic Maceration.—A 10-Gm. sample of the previously sized powder was placed in the treatment vessel (Fig. 1a). Eight milliliters of strong ammonia solution was added to the powder and mixed. Twenty-five milliliters of 2:1 ether-alcohol maceration fluid was then added. The sides of the treatment vessel were washed down with a few milliliters of solvent, the vessel was attached to the adjustable stand, and lowered into the vibrating oil bath of the previously tuned ultrasonic generator. The distance between the crystal face and treatment vessel was then adjusted for maximum turbulence of the maceration fluid. The maceration fluid reached boiling temperature after about five minutes of treatment. When this temperature was reached, the turbulence of the solution decreased noticeably. At the end of the desired exposure period, the slurry was removed and filtered. The moist drug was transferred to an extraction thimble and placed in the Soxhlet apparatus.

The maceration liquid, together with sufficient ether to extract the drug, was placed in the flask under the Soxhlet apparatus. The material was extracted for three hours and assayed as described by the U. S. P. XV (7). Duplicate trials of the ultrasonic maceration were performed in a progressively timed series of two, five, ten, fifteen, and twenty minutes. The results of these macerations and extractions are shown in Fig. 3.

The twenty-minute ultrasonic maceration was repeated with 10 samples and the powdered drug was collected and dried overnight at 105°. The 100 Gm. of dried drug were subjected to sieve separation. Some particle size increase was shown (Table 1). The extracts from three of the twenty-minute trials were assayed, and were found to contain 0.23% alkaloids.

Ultrasonic Extraction.—A 10-Gm. sample of the ground belladonna leaf was treated in the manner described for the ultrasonic maceration. The sample and the ammonia-ether-alcohol solvent was placed in the submerged type treatment vessel (Fig. 1b). The vessel was completely filled with ether, being careful to avoid bubbles in the vessel. The treatment vessel was attached to the adjustable stand and submerged in the oil bath of the previously tuned ultrasonic generator. The distance between the crystal face and the bottom of the treatment vessel was adjusted for maximum turbulence above the submerged treatment vessel and the sample was exposed for the desired length of time. The suspension was removed and filtered, the powdered drug washed with ether, and the washings added to the solvent. The total extract was assayed by the U. S. P. method (7). The above procedure was repeated with 5-Gm. samples. Duplicate experiments were conducted for each treatment period. The results of these extractions are shown in Fig. 2.

Twenty 5-Gm. samples were subjected to a twenty minute submerged ultrasonic treatment using the above procedure. The treated, powdered drug was collected and dried overnight at 105°. A sieve separation was done and the results indicated in Table I.

Chromatography of Ultrasonic Extracted Alkaloids.—An alkaloidal extract, obtained by U. S. P. XV procedures was used as the reference standard. The extract was treated according to the U. S. P.

XV assay procedure (7) to the point of heating the evaporated chloroform extract on the boiling water bath. The alkaloidal residue was then dissolved in 8 ml. of chloroform and transferred to a 10-ml. volumetric flask with sufficient chloroform to bring the volume to 10.0 ml. Circular chromatograms were prepared from Whatman No. 1 filter paper using Romeike's procedure (8). Twenty μl. of the chloroform solution containing approximately 0.086 mg. of atropine were spotted in two successive 10-μl. portions. After developing and drying the chromatograms, they were sprayed with Munier's modification of Dragendorff's reagent (12) and examined for zones of color. The only zone evident was that of atropine. Similar experiments were carried out with the twenty-minute ultrasonic maceration and ultrasonic extraction solvents. On the chromatograms from both of these extracts, only the atropine zones were evident.

DISCUSSION

Ultrasonic energy was applied to extraction of belladonna leaf U.S.P. The results obtained with regard to the methods used, indicate a possible explanation of the beneficial effect observed.

Increased agitation will speed extraction. Violent agitation was observed when a slurry of belladonna leaf in an ethereal solvent system was subjected to ultrasonic energy. This suggests one reason for speeded maceration and extraction. The tremendous acceleration imparted by ultrasonic energy indicates another reason. This acceleration of the particles would give rise to heating effects, and increased temperature will normally facilitate extraction. Cavitation took place during performance of ultrasonic maceration in the open treatment vessel, and possibly occurred during extraction in the closed vessel, but could not be observed. Collapse of the cavities formed during cavitation causes pressure to be built up locally and this, also, tends to accelerate extraction. Reducing particle size will normally speed extraction procedures. In the case of submerged ultrasonic extraction, a particle size reduction occurred. This may be another reason extraction was speeded.

Maceration by the described ultrasonic procedure caused an increase in particle size. Several explanations are possible. Apparently the surface treatment of the slurry did not permit as great an amount of ultrasonic energy to pass through due to damping of the waves at the upper liquid-air interface, as postulated by Newton and Kissel (9). This decreased energy in combination with the effect of cavitation is suggested as an answer. If a particle is subjected to pressure, as are the particles during cavitation, and the pressure suddenly released, the particle would tend to swell. Lack of sufficient energy to cause fragmentation would give the end result noted, a particle size increase. If the energy level is higher, as it apparently is in submerged treatment, the swelled particle may be disintegrated with resulting particle size reduction. Although a particle size increase was shown, maceration time was shortened considerably. The contribution of each of these effects has not been firmly established and further investigation is necessary to determine the actual mechanism of ultrasonic extraction.

Application of ultrasonic energy to drug extraction on an industrial scale would seem feasible. This has been accomplished in the brewing industry as previously mentioned (3, 4). Ultrasonic treatment gave more complete extraction in a shorter period of time than the official method. Results of these experiments indicate that submerged treatment is more efficient than surface treatment. Surface treatment for twelve minutes yielded 0.23% alkaloids, while submerged treatment for the same period of time gave 0.41% alkaloidal extraction. The submerged treatment would seem to be the method of choice for industrial scale and could probably be readily adapted to a "continuous flow" process.

SUMMARY

When the maceration slurry of belladonna leaf U. S. P. XV, is subjected to ultrasonic energy, the maceration time is shortened considerably. The eight-hour period (overnight) can be shortened to approximately twenty minutes and the subsequent Soxhlet extraction will yield an almost identical amount of alkaloids. The use of the described ultrasonic extraction procedures will yield a comparable amount of alkaloid in a greatly shortened time when compared with the official method.

The decomposition of the alkaloids by the ultrasonic waves and their accompanying effects was not evident under the conditions employed. Normal hydrolysis products were not present in an amount sufficient to be detected by the chromatographic method used.

Ultrasonic maceration with the surface treatment vessel resulted in some increase in particle size, but ultrasonic extraction in the submerged treatment vessel indicated a decrease in particle size.

The results obtained in these experiments indicate that the extraction of alkaloids of belladonna leaf U. S. P. is facilitated by the application of ultrasonic energy. The use of a submerged type treatment vessel may have commerical application.

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An Investigation of the Possible Potentiation of Ethinamate Activity by Chlorpromazine*

By WILLIAM R. McGRATH† and HOWARD J. JENKINS

Results of an attempt to determine the extent of the ability of chlorpromazine under specific circumstances to potentiate the hypnotic activity of ethinamate are presented. These results are compared with those pertaining to the extent of the ability of chlorpromazine to enhance ethinamate toxicity.

THE PREVAILING VIEW apparently supports a qualitative rather than a quantitative difference of action between sedative agents, such as the barbiturates, and the so-called tranquilizers. Chlorpromazine, one of the more widely used and investigated of the tranquilizers, has been found capable of potentiating narcotic activity and the effects of certain central nervous system depressants (1–3, 7–10). In spite of the number of reports in the literature with respect to chlorpromazine's ability to potentiate the activity of such agents, there appears to be little information as to the extent of this ability.

Coincident with the emersion of the tranquilizers has been the appearance of a group of agents called nonbarbiturate hypnotics. The study reported in this thesis was chiefly concerned with the ability of chlorpromazine to potentiate the hypnotic activity of these agents and, if this were demonstrable, with the extent of the potentiation. The object of the study was to provide information relative to the advisability of combination therapy involving a tranquilizer and a nonbarbiturate hypnotic with a view toward decreasing the dosage of the hypnotic agent.

EXPERIMENTAL

Two approaches were selected to determine the extent of the possible potentiation by chlorpromazine of the hypnosis of ethinamate, one of a series of aliphatic carbinols tested by Langdecker (4) and found to have hypnotic activity. In the first approach, the hypnotic dose of ethinamate for 50% of the injected animals (HD_{50}) was computed from results obtained upon injection of three dosage levels of the drug. Later, the HD_{50} of ethinamate when administered with chlorpromazine to the same animal sample, and finally the HD_{50} of ethinamate in the same animal sample when chlorpromazine was administered prior to ethinamate were determined. The ethinamate HD_{50} values representing combined effects were then compared with the HD_{50} value for ethinamate alone. The pretreatment procedure was suggested by Mattis (5).

In the second approach, prolongation of sleeping time was selected as an index of the extent of potentiation of ethinamate hypnosis by chlorpromazine. After the determination of the mean sleeping-time response of a group of animals to ethinamate, the ability of chlorpromazine, administered prior to and at the same time as ethinamate, to potentiate this mean response in the same animal group was determined.

The two drugs, because of their pharmacologic properties, lend themselves to this type of study. In therapeutic quantities, chlorpromazine exhibits no hypnotic activity and ethinamate hypnosis is rapid in onset and of brief duration (4, 6). Hence, prolongation of sleeping time is easily detected and is theoretically, at least, a potentiation index of value.

The original investigation plan recognized the need for a study of the combined effects of the two drugs with regard not only to hypnosis but also to blood pressure activity and acute and chronic toxicity. Since chlorpromazine possesses autonomic properties which are manifest as antihypertensive activity, it was considered possible that ethinamate, which has no such activity of its own in therapeutic amount, might potentiate the hypotensive effect of chlorpromazine. The study reported was concerned, however, only with the combined effects of the drugs on the hypnotic activity and the acute toxicity of ethinamate.

The test animals were male albino rats of the Carworth-Wister strain which weighed approximately 100 Gm. each at the beginning of the study and 275 Gm. at the end. The rats were maintained on Rockland rat diet and water, *ad libitum*, in a room whose temperature averaged 70° F. ± 5°.

Polyethylene glycol 200, reported to be a good ethinamate solvent (3), proved quite satisfactory as a vehicle. Distilled water was used as the solvent for chlorpromazine. All injections were made intraperitoneally into the lower left quadrant of the abdomen. The solutions were so prepared that the injection volume never exceeded 0.3 ml.

The loss of the righting reflex, the rat failing to right itself after being turned over on its back, constituted the end point of the test. In the rats that responded in this manner, the onset of action of ethinamate was rapid, usually within one to two minutes after the injection was completed. It was observed that those rats sleeping in response to ethinamate alone exhibited slow, regular, and deep respiration; whereas those sleeping in response to a combination of ethinamate and chlorpromazine exhibited very rapid and shallow respiration, with little or no apparent abdominal breathing.

The Litchfield-Wilcoxon (11) method was used for the statistical evaluation of the data of this part

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of the study. Also the significance of difference test was applied to the individual HD_{50}'s and these were found to differ significantly from each other and from the reference value of ethinamate alone.

At various intervals throughout the work the HD_{50} of ethinamate alone was checked to make sure that the sample's responsiveness to ethinamate had not changed appreciably.

At the outset of the study, an attempt was made to evaluate the potentiating ability of chlorpromazine in prolonging the sleeping time of rats responding to ethinamate hypnotic effect. Because of the rather wide deviation from the mean of the induced sleeping times in the early work, subsequent attempts were abandoned in favor of the reduction of HD_{50} method. The data of one of these sleeping-time determinations are included to show that chlorpromazine appears to prolong the sleeping time in ethinamate hypnosis. In this work all injections were made intraperitoneally. The data obtained with animals which received chlorpromazine ten minutes before ethinamate are reported since the previously presented results indicate that this pretreatment interval is optimal for the greatest potentiating effect in terms of ethinamate HD_{50} reduction.

The next portion of the work was undertaken to learn to what extent chlorpromazine would lower the LD_{50} of ethinamate. Male albino mice weighing approximately 20 Gm. were used as the test animals in the evaluation of toxicity potentiation. The animals were divided into three dosage groups of 10 animals each after which they were injected intraperitoneally with ethinamate alone. The results in terms of per cent response to each of the three dosage levels were plotted on log-probability paper, and the LD_{50} read from the plotted curve. The LD_{50} of ethinamate representing combination effects with chlorpromazine was determined by the same procedure, except that chlorpromazine in different dosage was administered either ten minutes prior to or at the same time as ethinamate. LD_{50}'s were computed according to the Litchfield-Wilcoxon method.

RESULTS AND DISCUSSION

The original HD_{50} of ethinamate alone and its confidence limits are listed in Table I along with all subsequently determined ethinamate HD_{50}'s, so that the original value can be compared with the latter. Figures 1 and 2 show, respectively, the effects of varying the interval between the administration of chlorpromazine and the administration of ethinamate and the combined effects of varying the dose plus the pretreatment interval of chlorpromazine on the per cent reduction of the ethinamate HD_{50}. The mean sleeping time of 30 rats which had been given 50 mg./Kg. of ethinamate was 26.1 minutes. The 19/20 confidence limits of this mean were found to be 9.22 minutes to 42.89 minutes. In the same animal group when 3 mg./Kg. of chlorpromazine was administered ten minutes before the above dose of ethinamate was given, the mean sleeping time was increased to 45.65 minutes. This mean represents an increase of 42.6%. The decrease in HD_{50} under similar experimental conditions was 38.8%. The rather wide confidence limits

TABLE I.—THE EFFECT OF VARIOUS COMBINATIONS WITH CHLORPROMAZINE ON THE ETHINAMATE HD_{50}

Procedure	HD_{50} Obtained	Reduction from Reference HD_{50}
Reference HD_{50} (ethinamate alone)	36.0 mg./Kg. (31.0–41.8 mg./Kg.) (19/20 Confidence limits)	..
Ethinamate and 3 mg./Kg. chlorpromazine administered together	27.5 mg./Kg. 27.5 mg./Kg.[a]	23.6
3 mg./Kg. chlorpromazine administered 10 minutes before ethinamate	22.0 mg./Kg. 21.0 mg./Kg.[a]	38.8
3 mg./Kg. chlorpromazine administered 20 minutes before ethinamate	25.0 mg./Kg. 26.0 mg./Kg.[a]	30.6
3 mg./Kg. chlorpromazine administered 30 minutes before ethinamate	29.0 mg./Kg. 30.0 mg./Kg.[a]	19.4
Ethinamate and 1 mg./Kg. chlorpromazine administered together	32.0 mg./Kg. 30.0 mg./Kg.[a]	11.1
1 mg./Kg. chlorpromazine administered 10 minutes before ethinamate	28.0 mg./Kg. 28.5 mg./Kg.[a]	22.2

[a] Duplicate determinations.

obtained, however, make this type of evaluation generally inadequate for determining the extent of potentiation.

The LD_{50} of ethinamate alone and in various combinations with chlorpromazine are listed in Table II. Figure 3 compares the per cent reduction of ethinamate HD_{50} and ethinamate LD_{50} by like doses of chlorpromazine administered at the same time that ethinamate was given. Table III shows the effect of various combinations with chlorpromazine on the therapeutic ratio of ethinamate. Table IV presents evidence of the fact that the HD_{50} of ethinamate alone differs significantly from the HD_{50} of ethinamate in each combination. It presents the same evidence of significant difference between the LD_{50} of ethinamate alone and the LD_{50} of ethinamate in each combination.

In order to determine that chlorpromazine was not exerting any hypnotic or lethal effect of its own, 5, 10, and 15 mg./Kg. doses, respectively, were administered to three groups of 20 rats each. No hypnotic or lethal effect was exhibited when chlorpromazine was administered in these amounts. Since these doses are up to 5 times the amount administered in the actual experimental work, it was assumed that chlorpromazine was not exerting any effect of its own.

Tests were also conducted in which 2 ml. of the

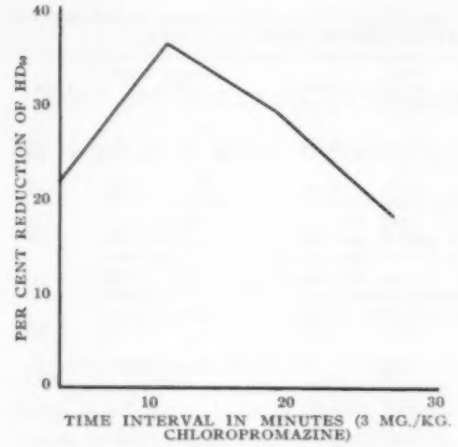

Fig. 1.—Effect of varying the time of pretreatment on the ability of chlorpromazine to reduce the HD_{50} of ethinamate.

TABLE II.—THE EFFECT OF VARIOUS COMBINATIONS WITH CHLORPROMAZINE ON THE ETHINAMATE LD_{50}

Procedure	LD_{50} Obtained,	Reduction from Reference LD_{50}, %
Reference LD_{50} (ethinamate alone)	324 (300–344.4) (19/20 confidence limits)	..
Ethinamate and 3 mg./Kg. chlorpromazine administered at the same time	270	16.6
3 mg./Kg. chlorpromazine administered 10 minutes before ethinamate	280	13.5
Ethinamate and 1 mg./Kg. chlorpromazine administered at the same time	320	1.2

TABLE III.—RATIOS OF ETHINAMATE LD_{50} TO HD_{50} IN VARIOUS COMBINATIONS WITH CHLORPROMAZINE (C. P. Z.)

Drug(s)	Pretreatment Interval, min.	LD_{50}/HD_{50} Ratio	LD_{50}/HD_{50} Index
Ethinamate	..	$\frac{324 \text{ mg./Kg.}}{36 \text{ mg./Kg.}}$	9.0
Ethinamate-3 mg./Kg. C. P. Z.	0	$\frac{270 \text{ mg./Kg.}}{27.5 \text{ mg./Kg.}}$	9.7
Ethinamate-3 mg./Kg. C. P. Z.	10	$\frac{280 \text{ mg./Kg.}}{22 \text{ mg./Kg.}}$	12.7
Ethinamate-1 mg./Kg. C. P. Z.	0	$\frac{320 \text{ mg./Kg.}}{32 \text{ mg./Kg.}}$	10.0

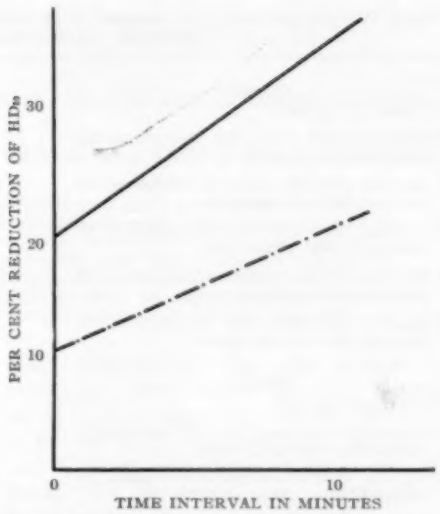

Fig. 2.—Dose as a factor in the ability of chlorpromazine to reduce the HD_{50} of ethinamate. —— 3 mg./Kg.; ———— 1 mg./Kg.

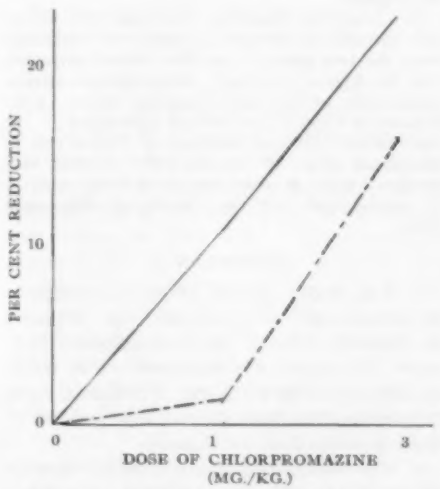

Fig. 3.—Ability of chlorpromazine to reduce the HD_{50} of ethinamate compared with its ability to reduce the LD_{50}. ———HD_{50}; — - — - — LD_{50}.

Carbowax solvent were administered to a group of 20 ·ats. In this volume, which represents almost 7 times the largest injection volume in the experimental work, the solvent seemed to be without biologic activity.

Essential criteria for the evaluation of combination therapy in this instance are the magnitude of the hypnosis-potentiating ability of the tranquilizer, indicated by the comparative effectiveness of the hypnotic administered alone, at the same time as or shortly after the tranquilizer; and the extent of the

TABLE IV.—SIGNIFICANCE OF DIFFERENCE BETWEEN THE HD_{50} (LD_{50}) OF ETHINAMATE ALONE AND THE HD_{50} (LD_{50}) OF ETHINAMATE IN COMBINATION

Procedure	HD_{50}, mg./Kg.	fHD_{50}	Potency Ratio* (P. R.)		f P.R.
Ethinamate (alone)	36.0	1.16	....		..
Ethinamate and 3 mg./Kg. chlorpromazine administered together	27.5	1.24	$\frac{36}{27.5}$	1.3	1.16
3 mg./Kg. chlorpromazine administered 10 minutes before ethinamate	22.0	1.28	$\frac{36}{22.0}$	1.63	1.34
3 mg./Kg. chlorpromazine administered 20 minutes before ethinamate	25.0	1.22	$\frac{36}{25}$	1.44	1.28
3 mg./Kg. chlorpromazine administered 30 minutes before ethinamate	29.0	1.14	$\frac{36}{29}$	1.24	1.20
1 mg./Kg. chlorpromazine administered 10 minutes before ethinamate	28.0	1.14	$\frac{36}{28}$	1.28	1.22
Procedure	LD_{50} mg./Kg.	fLD_{50}	Potency Ratio (P. R.)		f P.R.
Ethinamate (alone)	324	1.06	....		..
Ethinamate and 3 mg./Kg. chlorpromazine administered at same time	270	1.05	$\frac{324}{270}$	1.2	1.1
3 mg./Kg. chlorpromazine administered 10 minutes before ethinamate	280	1.16	$\frac{324}{280}$	1.15	1.14

* In Litchfield-Wilcoxon method if P. R. exceeds f P. R., HD_{50} (LD_{50}) values differ significantly.

ability of the tranquilizer to reduce the lethal dose of the hypnotic.

The study was therefore concerned with significant increase in hypnotic potency of ethinamate when the two agents were administered together, over the hypnotic potency of ethinamate administered alone, and also with significant increase in the duration of hypnotic activity of ethinamate in the combination over the duration of this action of ethinamate alone. It was likewise concerned with significant increase in the toxicity of ethinamate in the combination over the toxicity of ethinamate alone.

SUMMARY

1. The ability of one of the tranquilizers, chlorpromazine, to potentiate the hypnotic effectiveness of one of the nonbarbiturate hypnotics, ethinamate, was measured in two ways: by reduction of the HD_{50} dose of ethinamate and by increasing the mean sleeping time response of rats to a specific dose of ethinamate.

2. The potentiating ability of chlorpromazine with respect to ethinamate hypnotic activity was determined according to these indexes when the two drugs were administered at the same time, and also when ethinamate was administered at various times following chlorpromazine administration.

3. The data presented indicate that chlorpromazine is capable of potentiating ethinamate hypnotic effectiveness and, at the same time, point to the extent of this capacity, which seems to vary with the interval between the preadministration of chlorpromazine and the administration of ethinamate and, of course, with the dose of the potentiator.

4. Toxicity studies indicate that chlorpromazine potentiates the toxicity of ethinamate, although to a lesser extent than it potentiates the hypnotic effectiveness.

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Comparative Activities and Toxicities II.*

Sugar and Amino Acid Derivatives of Bis-(4-aminophenyl)-sulfone

By WILLIAM O. FOYE, WINTHROP E. LANGE, and EDWARD G. FELDMANN

The acute toxicities and antibacterial activities of the various sugar derivatives of bis-(4-aminophenyl)-sulfone have been compared with those of several α-amino acid derivatives. For this purpose, it was necessary to synthesize α-amino acid derivatives and to place the reported toxicity values for the sugar derivatives on a standard basis. It was found that the α-amino acid substituents were less effective in reducing the toxicity of bis-(4-aminophenyl)-sulfone than the sugars, but there was a comparable retention of antitubercular activity with both types of derivatives.

A COMPARISON of sugar and α-amino acid derivatives of nitro- and aminosalicylic acids has revealed that the α-amino acids are as effective as the sugar substituents in lowering toxicity and give a slightly better retention of antitubercular activity (1). In order to make a similar comparison between the corresponding derivatives of the toxic bis-(4-aminophenyl)-sulfone (2), the activities and toxicities of various sugar derivatives have been obtained from the literature, while it was necessary to synthesize and have tested several α-amino acid derivatives.

A reaction product of bis-(4-aminophenyl)-sulfone and glucose, of undetermined structure, was reported by Buttle (3) in 1938. This product, probably the bis-glucose anil, was described as having about half the activity of the parent substance against pneumococcal infections when given orally, and the lethal toxicity was listed as 50–100 mg. in 20-Gm. mice. Boyland (4), using Buttle's product, listed the toxic dose as ten times that of bis-(4-aminophenyl)-sulfone itself. Based on reported values (5) for the toxicity of bis-(4-aminophenyl)-sulfone (LD_{50} = 200–500 mg./Kg. in mice), Boyland's toxicity for the bis-glucose anil becomes approximately 2.5 Gm./Kg. for the LD_{50} in mice, which agrees with the lower limit of toxicity reported by Buttle for this compound.

The oral toxicity of the bis-glucose-sodium bisulfite addition product of bis-(4-aminophenyl)-sulfone (Promin) was listed by Banks and Gruhzit (6) in 1948. The LD_{50} for the parent sulfone was given as 12.6 mg. and for the addition product as 130 mg. per 20-Gm. mouse. These values may be extrapolated to give an LD_{50} of 630 mg./Kg. for the parent sulfone and an LD_{50} of 6.5 Gm./Kg. for Promin when given orally to mice. Although the LD_{50} value obtained for bis-(4-aminophenyl)-sulfone by these investigators is slightly larger than that listed by Smith or Raiziss (5), the sugar derivative again has a toxicity about one-tenth that of the parent compound. The therapeutic index, however, determined against β-hemolytic streptococci, remained unchanged in the case of Promin, where the lowest M. E. D. was also ten times that of the parent sulfone.

Sah (7) reported in 1949 the preparation of a condensation product of L-ascorbic acid with bis-(4-aminophenyl)-sulfone. The LD_{50} of this product was given as 987.7 ± 94.8 mg./Kg. in mice orally. In comparing this compound with bis-(4-aminophenyl)-sulfone and its sugar derivatives, Sah listed the following order of decreasing chronic toxicities: bis-(4-aminophenyl)-sulfone, a D-galacturonyl derivative, Promin, and the ascorbic acid derivative. This order is put in question somewhat by the acute toxicity of Promin obtained by Banks and Gruhzit (one-tenth that of the parent sulfone), since Sah states his ascorbic acid derivative to have one-fourth the toxicity of the parent sulfone (both values for mice orally). In a later publication of Kumler and Sah (8), however, the LD_{50} of the ascorbic acid derivative is reported as 1.0–2.6 Gm./Kg. (orally in mice) without explanation. The chemotherapeutic ratio of the bis-L-ascorbate compared to that of the parent sulfone was given as 3:2.

Costantino and Callerio (9) also made a comparison of various sugar derivatives of bis-(4-aminophenyl)-sulfone and included a galactoside (Tibatin). The "largest tolerated doses" in the mouse listed on a Gm./Kg. basis are as follows: bis-(4-aminophenyl)-sulfone, 0.5 Gm./Kg. (orally); the galactoside, 10 Gm./Kg. (intravenously and intraperitoneally); Promin, 5 Gm./Kg. (intravenously) and 7.5 Gm./Kg. (intraperitoneally). The toxicity of the parent sulfone agrees fairly well with the values reported by Smith (5) and by Banks and Gruhzit (6), although

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The authors are very grateful to Drs. K. K. Chen, R. C. Anderson, W. S. Boneice, and F. Komann of the Lilly Research Laboratories for providing the results of the toxicity determinations and antimicrobial testing.

different routes of administration were used. The toxicity of Promin also compares quite well with the previously reported value (6), and the ratio of toxicity of the parent sulfone to that of Promin is again ten to one. From this report, in addition, it appears that the galactose derivative would be one-half to three-fourths as toxic as Promin when administered orally.

Despite the confusion resulting from different methods of toxicity determinations, different means of reporting them, and in some cases the use of uncharacterized products, it has been possible by extrapolation of data to make a direct comparison of the toxicities of sugar derivatives of bis-(4-aminophenyl)-sulfone using a standard basis. This is shown in Table I.

The use of α-amino acids for the purpose of detoxication has not been investigated seriously, although Parrod (10) has reported the preparation of the mono- and bis-*dl*-alanyl derivatives of bis-(4-aminophenyl)-sulfone. The mono derivative showed both one-third the toxicity in the mouse and one-third the activity of the parent sulfone toward tuberculosis bacilli, while the bis derivative was inactive. This result indicates that at least one free amino group is necessary on the aromatic rings for bacteriostatic activity and that side-chain amino groups are ineffective.

Several α-amino acid derivatives were therefore synthesized for further comparison, namely the bis-4-glycyl, the mono-4-phenylalanyl, and the bis-4-glycylglycyl derivatives, the first two in sufficient quantities for biologic testing. The bis-glycyl derivative was found by Dr. R. C. Anderson to have an LD_{50} of about 800 mg./Kg. in mice intraperitoneally, while the mono-*dl*-phenylalanyl derivative caused no deaths at a dose of 1,666 mg./Kg. The bis-glycyl derivative was screened against 38 [illegible] organisms and found to inhibit none of them at a concentration of 200 μg./ml., using the agar dilution technique. Both the α-amino acid derivatives showed positive activity in experimental mouse tuberculosis, however, of the same order of activity as that from bis-(4-aminophenyl)-sulfone itself. These biological determinations were carried out at the Lilly Research Laboratories; and the toxicities of the known α-amino acid derivatives are listed in Table II.

TABLE I.—ACUTE ORAL TOXICITIES OF SUGAR DERIVATIVES OF BIS-(4-AMINOPHENYL)-SULFONE

Bis-(4-aminophenyl)-sulfone Derivative	LD_{50} (Mice), Gm./Kg.	Ref.
Bis-(4-aminophenyl)-sulfone	0.5	3–5
Bis-glucose anil	2.5–5.0	3,4
Bis-glucose-sodium bisulfite addition compd.	6.5	6,9
Bis-L-ascorbic acid deriv.	1.0–2.6	7,8
Bis-galactoside	9–12	9

TABLE II.—ACUTE TOXICITIES OF α-AMINO ACID DERIVATIVES OF BIS-(4-AMINOPHENYL)-SULFONE

Bis-(4-aminophenyl)-sulfone Derivative	LD_{50} (Mice), Gm./Kg.	Mode of Administration
Mono-*dl*-alanyl amide	1.5	Oral
Bis-glycyl amide	0.8[a]	Intraperitoneal
Mono-*dl*-phenylalanyl amide	>1.7[a]	Intraperitoneal

[a] Determined at the Lilly Research Laboratories by Dr. R. C. Anderson and W. S. Boneice.

METHOD OF PREPARATION

The general synthetic procedure followed for the α-amino acid derivatives involved the acylation of bis-(4-aminophenyl)-sulfone with α-phthalimido acid chlorides followed by hydrazinolysis of the phthaloyl groups. Attempts to carry out acylation with phthalimidoacetyl chloride in refluxing benzene-pyridine solution failed, and in glacial acetic acid with sodium acetate, only a 23% yield of desired bis-(4-phthalimidoacetamidophenyl)-sulfone was obtained. Using pyridine as solvent at 0°, however, to form the acyl pyridinium halide, a 76% yield was obtained, and the decomposition previously noted was minimized.

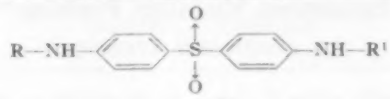
Diacylation involving both amino groups resulted from the reaction with phthalimidoacetyl chloride, but in the reaction with N-phthaloylphenylalanyl chloride, only mono-acylated product was isolated, in 71% yield. In the preparation of the glycylglycyl derivative, phthalimidoacetyl chloride was allowed to react with bis-(4-glycylaminophenyl)-sulfone. This procedure avoided formation of the acid chloride of a dipeptide, which easily undergoes azlactone formation. Although the bis-glycyl derivative was sparingly soluble in cold pyridine, a fair yield of the bis-phthaloylglycylglycyl derivative resulted. Removal of the phthaloyl groups with hydrazine hydrate gave yields of 80–93% in general. The physical properties of the sulfones prepared are listed in Table III.

EXPERIMENTAL

Bis-(4-phthalimidoacetamidophenyl)-sulfone.—In a three-necked flask equipped with an Ace Trubore stirrer was placed 2.5 Gm. (0.01 mole) of bis-(4-aminophenyl)-sulfone (Bios Laboratories) and 50 ml. of dry pyridine. The solution was cooled to 0°, and 4.5 Gm. (0.02 mole) of phthalimidoacetyl chloride (11) was added during ten minutes with stirring. The resulting solution was stirred for four hours at 0° and then poured into 250 ml. of cold water containing 25 ml. of sulfuric acid. The white precipitate was collected, washed with water, and recrystallized twice from glacial acetic acid, giving 4.7 Gm. (76%) of product which did not melt below 300°.

Bis-(4-aminoacetamidophenyl)-sulfone.—A mixture of 2.0 Gm. (0.003 mole) of bis-(4-phthalimidoacetamidophenyl)-sulfone, 0.4 Gm. (0.008 mole) of 85% hydrazine hydrate, and 20 ml. of water was stirred magnetically at 100° for three hours. The

TABLE III.—α-AMINO ACID AMIDES OF BIS-(4-AMINOPHENYL)-SULFONE



R	R¹	Melting Point[a]	Formula	Analyses, %	
				Calcd.	Found[b]
Phthaloylglycyl	Phthaloylglycyl	>300°	$C_{32}H_{22}N_4O_8S$	C 61.67	61.45
				H 3.56	3.59
Glycyl	Glycyl	225–227°	$C_{16}H_{18}N_4O_4S$	C 53.02	53.39
				H 5.01	5.51
Phthaloylphenylalanyl	H	234–236°	$C_{39}H_{28}N_4O_6S$	C 66.27	66.74
				H 4.41	4.77
Phenylalanyl	H	176–178°	$C_{27}H_{24}N_4O_2S$	C 63.77	63.76
				H 5.35	5.53
Phthaloylglycylglycyl	Phthaloylglycylglycyl	255–257°	$C_{36}H_{28}N_6O_{10}S$	C 58.69	58.30
				H 3.83	3.81
Glycylglycyl	Glycylglycyl	201–204° (dec.)	$C_{20}H_{24}N_6O_6S$	C 50.41	51.05
				H 5.08	4.84

[a] The melting points were taken on a Fisher-Johns block and are uncorrected.
[b] The analyses were carried out by Weiler and Strauss, Oxford, England.

resulting solution was cooled and acidified with 20% hydrochloric acid. The precipitated phthaloyl hydrazide was removed, and the filtrate was made basic with 10% sodium hydroxide solution. The resulting crystalline solid was isolated, dried, and recrystallized from absolute ethanol, yielding 1.0 Gm. (93%) of product which melted at 225–227°.

4-Aminophenyl-4′-(α-phthalimido-β-phenylpropionamidophenyl)-sulfone.—A solution of 2.5 Gm. (0.01 mole) of bis-(4-aminophenyl)-sulfone and 50 ml. of dry pyridine was made by slight warming, and then cooled to 0°. The solution was treated with 6.4 Gm. (0.02 mole) of *dl*-α-phthalimido-β-phenylpropionyl chloride (11) in the manner described above, and the white solid obtained was recrystallized from acetone-Skellysolve B solution. The yield was 3.7 Gm. (71%) of product melting at 234–236°. A positive test for primary amino group was observed by the diazotization procedure.

4-Aminophenyl-4′-(α-amino-β-phenylpropionamidophenyl)sulfone.—The derivative described in the preceding paragraph (3.7 Gm., 0.007 mole) was treated with 85% hydrazine hydrate (0.5 Gm., 0.01 mole) and 25 ml. of water as previously described. The resulting white, crystalline solid was recrystallized from 95% ethanol, and 2.4 Gm. (89%) of product was obtained which melted at 176–178°.

Bis-(4-phthalimidoacetamidoacetamidophenyl)-sulfone.—A solution of 2.5 Gm. (0.007 mole) of bis-(4-aminoacetamidophenyl)-sulfone in 75 ml. of dry pyridine was treated at 0° with 3.2 Gm. (0.014 mole) of phthalimidoacetyl chloride and stirred for six hours at 0° and for two hours while warming to room temperature. After the solution was poured into cold water (300 ml.) containing 40 ml. of sulfuric acid, a white solid was collected, washed with water, and recrystallized from absolute ethanol. A yield of 2.0 Gm. (38%) of product was obtained which melted at 255–257°.

Bis-(4-aminoacetamidoacetamidophenyl)-sulfone.—The hydrazinolysis already described was carried out on the preceding product, and a white, crystalline solid was obtained in 80% yield after recrystallization from absolute ethanol; m. p. 201–204° with decomposition.

SUMMARY

1. Several α-amino acid amides of bis-(4-aminophenyl)-sulfone, namely the bis-glycyl, the mono-phenylalanyl, and the bis-glycylglycyl, have been prepared. The use of α-phthalimidoacyl chlorides was suitable for this purpose.

2. Acute toxicity values for the sugar derivatives of bis-(4-aminophenyl)-sulfone reported in the literature have been expressed as oral LD_{50} values for mice and compared.

3. Acute toxicity values and antibacterial activities of the α-amino acid derivatives have been obtained. Whereas no *in vitro* activity against microorganisms was shown by the bis-glycyl derivative, both the mono- and bis-α-amino acid derivatives showed *in vivo* antitubercular activity on a par with the parent sulfone.

4. The α-amino acid derivatives do not lower the toxicity of bis-(4-aminophenyl)-sulfone to the extent that the sugar derivatives do, but they show a comparable retention of activity against experimental tuberculosis in mice.

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A Note on Several New Alkaloids from *Vinca rosea* Linn. I: Leurosine, Virosine, Perivine*

By GORDON H. SVOBODA

Vinca rosea Linn. (*Catharanthus roseus G. Don., Lochnera rosea* (Linn.) Reichb.) is an erect everblooming pubescent herb or subshrub, 1–2 feet high. It is cosmopolitan in the tropics and is cultivated in gardens in many areas.[1]

Several alkaloids have been recently isolated from this plant (1–4). The isolation of three new alkaloids, leurosine, virosine, and perivine,[2] is herein reported. Extraction of the crude drug was accomplished by intimately mixing the dried whole plant with a solution of 2% tartaric acid. The weak bases were extracted with benzene. After partial purification by the usual acid-base treatment, the crude alkaloidal mixture was chromatographed on alumina. This weak base fraction yielded tetrahydroalstonine, tetrahydroserpentine, leurosine, and virosine.

Extraction of the drug with benzene, after it was made alkaline with gaseous ammonia, yielded the stronger bases. Column chromatography of this alkaloidal mixture yielded perivine and lochnerine.

Pertinent physical data of these new alkaloids are as follows:

Leurosine.—Leurosine crystallizes with 6 molecules of water; $[\alpha]_D^{25}$ + 59.80 ($CHCl_3$); m. p. 200–203° (decompn.). Elemental analysis gives the following values: C, 68.27, 68.88, 68.42, 68.69; H, 6.71, 7.28, 7.07, 7.12; N, 6.83, 7.00, 7.09; OCH_3, 13.58; (N)CH_3, 2.28; (C) CH_3, 5.39. The actual empirical formula of leurosine has not as yet been completely defined. Work is under way to determine its structure.

The ultraviolet spectrum in ethanol shows maxima at 213 and 261 mμ with shoulders at 287, 294, and 310 mμ; log E(1%, 1 cm.) at 213 mμ = 2.76, log E(1%, 1 cm.) at 261 mμ = 2.24. The ultraviolet spectrum in acidic ethanol shows maxima at 214 and 266 mμ with shoulders at 285, 294, and 310 mμ; log E(1%, 1 cm.) at 214 mμ = 2.80, log E(1%, 1 cm.) at 266 = 2.27.

The infrared spectrum of a chloroform solution of the hexahydrate shows a weak band at 2.90 μ, suggesting an NH grouping. The intense bands occurring at 5.72 and 8.1 μ probably indicate the presence of one or more ester groupings in the molecule. Other bands occur at 2.80, 3.33, 3.39, 6.16, 6.63, 6.83, 6.95, 7.26, 7.49, 8.64, 8.71, 8.82, 8.91, 9.02, 9.15, 9.39, 9.59, 10.75, and 11.10 μ.

When chromatographed in isobutanol saturated with water on No. 1 Whatman paper saturated with pH 3.0 buffer, leurosine exhibits a dark spot at R_f 0.38–0.42 under short wavelength ultraviolet radiation.

Virosine.—M. p. 257–262° (decompn.). Elemental analysis, C, 68.88; H, 6.81; N, 7.43; OCH_3, 8.40; (N)CH_3, 0; (C)CH_3, 3.07. The ultraviolet spectrum shows maxima at 226 and 270 mμ, with shoulders at 280 and 292 mμ; log E(1%, 1 cm.) at 226 mμ = 2.88, log E(1%, 1 cm.) at 270 mμ = 2.33. The infrared spectrum of a chloroform solution shows characteristic bands at 2.81, 3.03, 3.43, 5.67, 6.88, 7.93, 8.78, 9.07, 9.20, 9.36, 9.52, 9.94, 10.55 μ. Virosine, as leurosine, exhibits a dark spot under short wavelength ultraviolet radiation on a paper strip; R_f 0.5.

Perivine.—M. p. 180–181°. Elemental analysis, C, 69.42, 69.72; H, 6.49, 6.45; N, 7.48, 7.41; OCH_3, 8.63; (N)CH_3, 1.82; (C)CH_3, 3.78. The ultraviolet spectrum shows a maximum at 314 mμ and shoulders at 226 and 240 mμ; log E(1%, 1 cm.) at 314 mμ = 2.67. The infrared spectrum of a chloroform solution shows characteristic bands at 2.90, 3.40, 5.79, 6.07, 6.34, 6.50, 6.89, 6.96, 7.52, 7.68, 8.58, 8.80, 8.95, 9.08, 9.46, 9.82, 10.63 μ. Perivine exhibits a bright blue fluorescence under short wavelength ultraviolet radiation on a paper strip; R_f 0.60.

Detailed methods of obtaining leurosine and studies on structure determination will be discussed at a later date, as will the biological activities presently being evaluated, primarily the effect of leurosine on experimental leukemias.

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The author is grateful to Messrs. R. J. Armstrong and A. T. Oliver for laboratory assistance, to Dr. H. E. Boaz and Mr. P. Landis for infrared data, to Mr. L. Howard for ultraviolet data, to Mr. H. Bird and Mrs. N. Cone for paper chromatographic studies, to Messrs. W. L. Brown, R. Hughes, H. L. Hunter, and G. M. Maciak for microanalytical data, and to Drs. M. Gorman and N. Neuss for valuable suggestions offered throughout the course of this investigation.

[1] The drug used in this study was obtained from the Meer Corp. and was botanically authenticated by Dr. V. H. Madis.

[2] Since the isolation of these alkaloids, it has been learned that Drs. R. L. Noble, C. T. Beer, and J. H. Cutts have reported the isolation of another new alkaloid from this plant (N. Y. Acad. Sci., in press), which they call vincaleucoblastine. The comparison of the physical properties of these compounds, including paper chromatographic behavior, shows them to be different. I should like to thank Dr. Noble and his associates for an authentic sample of vincaleucoblastine sulfate.

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Book Notices

The Impact of the Antibiotics on Medicine and Society. Edited by Iago Galdston. International Universities Press, Inc., New York, 1958. x + 222 pp. 14 × 21.5 cm. Price $5.

This monograph, the second published by the Institute of Social and Historical Medicine of the New York Academy of Medicine, covers a wide field starting with "The Microbiology of the Soil and the Antibiotics," by S. A. Waksman, to "The Effects of the Antibiotics on Society," by L. B. Hobson. Some of the other topics are: historical surveys of early antibacterial and antibiotic concepts, mass production of antibiotics, application to plant and livestock industry, and the extent of their impact on clinical medicine. No index is included.

Current Drug Handbook 1958. By Mary W. Falconer and H. Robert Patterson. W. B. Saunders Company, Philadelphia, 1958. viii + 157 pp. 16 × 23.5 cm.

This book is a tabulation of about 1,000 drugs in current use, grouped according to pharmacological or therapeutic properties. The information included is given under the column headings: names and general information (includes toxicity and treatment for poisoning), preparations, dosage, administration, and remarks on use. The difficulty in keeping such a listing current is indicated by the fact that N. N. R. 1956 is cited as a source of material in addition to U. S. P. XV and N. F. X. An index of the drugs listed is appended.

Aliphatic Fluorine Compounds. By A. M. Lovelace, Douglas A. Rausch, and William Postelnek. Reinhold Publishing Corporation, New York, 1958. x + 370 pp. 15 × 23 cm. Price $12.50.

This book is one of the American Chemical Society monographs. It covers the preparation and properties of all reported organic fluorine compounds other than the aromatic compounds. Each chapter is devoted to a specific class or classes of fluoroorganic compounds such as alkanes, alkenes, alcohols, etc. Physical properties and references are included in the 61 tables of different groups of compounds. References are given at the end of each chapter, and an index is appended.

American Drug Index 1958. By Charles O. Wilson and Tony Everett Jones. J. B. Lippincott Co., Philadelphia, 1958. 716 pp. 14 × 21 cm. Price $5.

The third edition (1958) of this very useful compilation of generic, trade, and chemical names and composition of drugs and dosage forms, with indicated uses of pharmaceuticals and chemicals, continues the comprehensive coverage of this constantly expanding field. The 1958 edition's 716 pages of condensed information indicates the added material when compared with the 650 pages in 1957 and 576 pages in 1956.

An Introduction to Pharmacology and Therapeutics. 9th ed. By J. A. Gunn. Oxford University Press, London, 1958. ix + 327 pp. 12 × 18.5 cm. Price $4.25.

This introductory textbook had not been revised since 1948. The author notes that the medical practitioner finds it hard to keep up with the rapid and continuous advances in therapeutics; that a student faces a list of outlandish names with which he must associate pharmacological and therapeutical properties. As a discriminatory basis he uses the B. P. and U. S. P. for guidance. The most substantial additions in this revision of the text have been made to antibiotics, hormones, autonomic drugs, anesthetics, analgesics, sedatives, and antihistamines. Noting under "Sedatives" that certain drugs are referred to by the seductive name of "tranquilizers," the author writes: "Their permanent value in therapeutics is not still precisely established. It can be expected that their indiscriminate use will not be without hazard."

The Atomic Age and Our Biological Future. By H. V. Brønsted. Translated by E. M. Huggard. Philosophical Library, New York, 1957. xiv + 80 pp. 12 × 18.5 cm. Price $2.75.

The aim of this pocket-size book is to describe the biological, and particularly the genetical, effects that might result unless rigorous control of atmospheric radiation is ensured.

Fat Consumption and Coronary Disease: The Evolutionary Answer to this Problem. By T. L. Cleave. Philosophical Library, New York, 1958. 40 pp. 12 × 18.5 cm. Price $2.50.

The author suggests two main rules in eating: Eat foods in their natural state (permits simple cooking). Eat foods in strict proportion to the appetite (mixtures only in accordance with personal tastes).

Our Nuclear Adventure, Its Possibilities and Perils. By D. G. Arnott. Philosophical Library, New York, 1958. xi + 170 pp. 14 × 22 cm. Price $6.00.

This small book is a review of basic information pertaining to nuclear reactions and the related behavior of mankind with this new force.

General Biochemistry. 2nd ed. By Joseph S. Fruton and Sofia Simmonds. John Wiley & Sons, Inc., New York, 1958. x + 1077 pp. 15 × 22.5 cm. Price $18.

This book presents a revision of the first edition which was reviewed in THIS JOURNAL, **42,** 507(1953). The style and arrangement has been retained, and a new chapter on Alternative pathways of carbohydrate metabolism has been added. It includes many references and a good index is appended. The book is intended as a textbook which presents the structure of modern biochemistry from a general point of view, and it is well done.

Vitamin B_{12}. Merck Service Bulletin. Merck and Co., Inc., Chemical Division, Rahway, New Jersey, 1958. 193 pp. 15 × 23 cm.

This latest volume in the series of Merck Service Bulletins presents a comprehensive coverage of information on vitamin B_{12}. It is directed particularly to the pharmaceutical manufacturer, but it contains much information of interest to others in pharmacy and related fields. Author and subject indexes are appended.

Czechoslovak Fine Chemicals Standards. Vol. 2. M. Nedeljak, editor. Chemapol, Praha II, Czechoslovakia, 1957. 444 pp. 12 × 21 cm.

This book (in English) is published by Chemapol, a foreign trade corporation for the import and export of chemical products and raw materials. It is the revised edition of Vol. 1, which was reviewed in THIS JOURNAL, **45,** 820(1956).

Cahiers de Synthèse Organique. Vol. 4. By Jean Mathieu and Andre Allais. Masson et Cie., 120, Boulevard Saint-Germain, Paris 6, France, 1958. 272 pp. 15 × 22 cm. Price 5500 F.

This fourth volume continues the series relative to the elaboration of molecular structures. It includes acylation in aliphatic and aromatic series, and bifunctional condensations. Each chapter presents a graphic synopsis; a discussion of the related principles; descriptions of the mechanisms, applications, and reactions; and a tabulation of starting materials, products obtained, condensing agents and solvents, yields, and references. A recapitulative table of the functional groups and the created arrangements covering vols. 1 to 4 are included.

Principles of Research in Biology and Medicine. By Dwight J. Ingle. J. B. Lippincott Company, Philadelphia, 1958. xv + 123 pp. 15 × 23 cm. Price $4.75.

This book is directed to students who are preparing for or are beginning research in microbiology and medicine. It is written in a style that should hold the student's interest while informing him of the proper mental approach to scientific experimentation in general and to the selected field in particular. The author states: "The student of truth must also study error,. . . ." and he gives 28 fallacies that apply to experimentation. It is a good book for any experimenter.

The Chemistry and Chemotherapy of Tuberculosis. 3rd ed. By Esmond R. Long. The Williams & Wilkins Company, Baltimore, 1958. xviii + 450 pp. 15 × 23 cm. Price $12.

This third edition follows the style and chapter arrangement of the second edition but the text has been rewritten to include the many recent advances in this field. Section I on Chemistry of Tubercle Bacilli covers all aspects from structure and staining to biologic activity of components. Section II includes nine chapters under Chemical Changes in the Tuberculous Host, and Section III includes eleven chapters on the Chemotherapy of Tuberculosis. Each chapter has its references and a good index is appended.

Veterinary Anaesthesia. 4th ed. By John G. Wright. The Williams & Wilkins Company, Baltimore, 1957. xiii + 317 pp. 14 × 22 cm. Price $5.75.

This fourth edition of a book which apparently has had wide use in Great Britain since 1941 has been brought up to date and represents the latest accepted procedures in presurgical and surgical anesthesia in farm animals, pets, and laboratory animals. The text is divided into 33 chapters and an index is appended.

Dynamic Anatomy and Physiology. By L. L. Langley, E. Cheraskin, and Ruth Sleeper. McGraw-Hill Book Company, Inc., New York, 1958. viii + 719 pp. 15.5 × 23 cm. Price $6.

This textbook presents a concise course on anatomy with the study of function or physiology integrated into the instructive requirements for junior college or college courses for nursing students. The text has many illustrations and includes a color atlas of the human body. An index is appended.

Clinical Enzymology. Edited by Gustav J. Martin. Little, Brown & Company, Boston, 1958. viii + 241 pp. 15 × 23.5 cm. Price $6.

This book presents the case for the parenteral administration of enzymes. The text is divided under the headings: Protein biology, Chemistry of enzymes used clinically, Biochemistry of enzymes used clinically, Parenteral use of enzymes in medicine, Diagnostic use of enzymes, Polymerases in biology, and Recapitulation and perspectives for clinical enzymology. References are given for each chapter and a subject index is appended.

Methods in Medical Research. Vol. 7. Warren V. Warren, editor-in-chief. The Year Book Publishers, Inc., Chicago, 1958. xiii + 237 pp. 14 × 21.5 cm. Price $7.50.

This volume, the seventh in the series, is divided into four main sections in which the contributed articles were grouped by the associate editors under the headings: Chemical investigation of muscular tissues, Hemodynamic methods—heart and lungs, Methods for the study of human leukocytes, and Methods for study of the histology and cytology of the retina. References are given for each article, and subject and name indexes are appended.

Ion Exchange Resins. 2nd ed. By Robert Kunin. John Wiley & Sons, Inc., New York, 1958. xiii + 466 pp. 15 × 23 cm. Price $11.

The second edition of this book is designed to serve the purpose of an introductory text and applications guide, just as was the first edition, which was reviewed in THIS JOURNAL, **40,** 219(1951). However, many of the developments have grown to such importance during the last eight years that whole chapters are devoted to them instead of the sentence or paragraph in the first edition, e. g., permselective membranes, hydrometallurgy, sugar refining, and specific exchange resins. The alphabetical list of numbered references (referred to by number in the text) includes 1,170 entries. Tabulated data, as well as author and subject indexes, are appended.

Infrared Absorption Spectra of Steroids. An Atlas. Vol. 2. By Glyn Roberts, Beatrice S. Gallagher, and R. Norman Jones. Interscience Publishers, Inc., New York, 1958. viii + 95 numbered pages and an atlas of 451 spectra on unnumbered pages. 15 × 23 cm. Price $20.

This second volume follows the style and arrangement of the first volume which was reviewed in THIS JOURNAL, **43,** 384(1954). The introductory portion is covered in 19 pages, and is followed by a table of characteristic group frequencies which occupies the next 19 pages. Spectra of 760 substances occupy most of the text, with a functional index and an alphabetical index of the charts at the end of this section.

Exposés Annuels de Biochemie Médicale (1957). Vol. 19. Published under the direction of P. Boulanger, M.-F. Jayle, J. Roche, and J. Polonovski (Secretariat). Masson et Cie, Editeurs, Libraires de L'Academie de Médecine, 120, Boulevard Saint-Germain, Paris-vi^e, 1957. 239 pp. 16.5 × 25 cm. Price 3,000 fr.

This revue (in French) of the biochemical medical advances is the nineteenth in the series and includes reports on: Some new aspects of the clinical study of plasma proteins, Macroglobulins and macroglobulinemias, Dysproteinemias, C-Reactive-protein Seric lipoproteins, Steatorrhea, Venomous substance from *Amanita phalloides,* Component amino-acids of tissues and body fluids, Thermodynamic study of immunologic reactions, Aldosterone, Recent progress in the chemistry and physiologic reaction of steroids, and Recent studies on steroidogenesis in human gonads. An index of authors, with the titles of their reports that appeared in volumes 1–19, is appended.

5-Hydroxytryptamine. Edited by G. P. Lewis. Pergamon Press, New York, 1958. xvii + 253 pp. 14 × 21.5 cm. Price $9.50.

This book is a compilation of the papers presented, and discussions of them, at a symposium on 5-hydroxytryptamine. The occurrence, metabolism, and the actions of this compound in areas affecting cardiovascular actions, nerve endings and ganglia, carcinoids, anaphylaxis, and gastric secretion are covered. Peripheral antagonists of 5-hydroxytryptamine, and its central actions and antagonists to them are reported. The general discussion covers occurrence and metabolism, peripheral actions, and central actions.

Standard Methods of Clinical Chemistry. Vol. 2. By the American Association of Clinical Chemists. Davis Seligson, editor-in-chief. Academic Press, Inc., New York, 1958. xiii + 217 pp. 15 × 23 cm. Price $5.50.

The explanation and description of tested clinical analyses in this volume follows the same style that was used in Vol. 1, which was reviewed in THIS JOURNAL, **42,** 706(1953). Subjects in Vol. 2 include: Calcium (complexometric), Cephalin-cholesterol flocculation test, Chloride, Cholesterol in serum, Total fatty acids in stool, Gamma globulin in serum, Hemoglobin, Free and conjugated 17-hydroxycorticosteroids in plasma, Iron in serum, 17-Ketosteroids in urine, Pancreatitis-lipase, Nitrogen by the Kjeldahl method, Nonprotein nitrogen, Determination of blood pH, Alkaline and acid phosphatase, Phosphatides in plasma, Porphyrins in urine, Protein-bound iodine in serum, Sodium and potassium by flame photometry, Sulfobromophthalein (BSP) in serum, and Urobilinogen in urine and feces. References are given after each subject, and author and subject indexes are appended.

The Year Book of Drug Therapy. 1957–1958 Series. By Harry Beckman. The Year Book Publishers Inc., Chicago, 1958. 518 pp. 13 × 19.5 cm. Price $7.50.

This annual review of medical literature represents the careful and complete coverage and selection by Dr. Beckman that has prevailed throughout this series. In an opening editorial, Dr. Beckman irately denounces some of the promotional activities associated with new drugs. The style and format of the series is maintained and the usual good author and subject indexes are included.

Methods of Biochemical Analysis. Vol. 6. Edited by David Glick. Interscience Publishers, New York, 1958. ix + 358 pp. 15.5 × 23 cm. Price $8.50.

This sixth volume of the series emphasizes methodology and instrumentation, with each chapter presented with a background discussion and review, followed by a critical evaluation of the various approaches and a presentation of the procedural details of the method or methods recommended by the author. The subjects covered are: New developments in the chemical determination of nucleic acids, The microbiological assay of nucleic acids and their derivatives, The determination of formaldehyde and serine in biological systems, New methods for purification and separation of purines, Assay of serotonin and related metabolites, enzymes and drugs, Determination of transaminase, Glycolipide determination, Methods for the determination of thiamine, Rapid electrophoresis in density gradients combined with pH and/or conductivity gradients, and reference author and subject indexes for this volume and cumulative indexes of contributors and subjects covered in volumes 1–6.

Ocular Allergy. By Frederick H. Theodore and Abraham Schlossman. The Williams & Wilkins Company, Baltimore, 1958. xviii + 420 pp. 15 × 23 cm. Price $12.

This book is directed primarily to the practicing ophthalmologist and allergist, but it includes much useful information for the dermatologist. Allergenic actions of cosmetics and drugs are reviewed, and, with reference to ophthalmic medicaments causing ocular eczema, it is stated: "Most of the time however, ointments, especially ones used to relieve itching or irritation of the eyelids, such as those containing anesthetics, antihistaminics or even corticosteroids, are the prime offenders. Even the mild ointment bases themselves may cause the reaction." Difficulties in diagnosis of ocular allergies are discussed. The text is illustrated and includes many references. An index is appended.

Pharmacology in Medicine. 2nd ed. Edited by Victor A. Drill. McGraw-Hill Book Company, Inc., New York, 1958. xi + 1243 pp. 21.5 × 28 cm. Price $19.50.

This second edition of a very good collaborative textbook designed for students and practitioners of medicine is presented in the same manner and style as the first edition, which was reviewed in THIS JOURNAL, **44**, 255(1955). The text material has been revised to include later information, and a new chapter on Drugs Affecting Behavior has been added. Chapter 40 was included as an Addendum in order to incorporate the recently marketed oral diuretic chlorothiazide (Diuril). The second edition merits the high opinion expressed for the original publication.

The Pasteur Fermentation Centennial 1857–1957. A Scientific Symposium edited by Editorial Projects, Inc. Chas. Pfizer & Co., Inc., New York, 1958. xiii + 207 pp. 15 × 23 cm.

This book is the record of the conference held to memorialize the publication by Pasteur, in 1857, of his paper showing that fermentation of lactic acid was caused by microorganisms. Included are symposia on: Cellular and biochemical interplay between host and parasite, New developments and prospects in fermentation chemicals, and Trends in antibiotic research. Bibliographies of all papers are appended, but a general index is omitted. The reports are interesting and instructive.

Federal Food, Drug, and Cosmetic Act, Judicial and Administrative Record, 1953–1957. Vincent A. Kleinfeld and Charles Wesley Dunn. Commerce Clearing House, Inc., Chicago, 1958. xiv + 1127 pp. 16.5 × 24.5 cm. Price $25.

This volume is the fourth book by Kleinfeld and Dunn on the Federal Food, Drug, and Cosmetic Act, compiling its amendment, administration, and judicial record from 1953 to 1958; and also the official annual reports on that Act. The three earlier volumes covered the periods 1938–1949, 1945–1950, and 1951–1952, and were reviewed in THIS JOURNAL, **40**, 304(1951); **41**, 51(1952); and **42**, 507(1953).

Their style and arrangement for clear and thorough coverage of the material established a pattern which has been very satisfactory, particularly for rapid reference, and it is followed in the present volume.

The book is divided into four major portions: (a) opinions and decisions, full texts of the Federal Food, Drug, and Cosmetic Act with amendments and principal regulations; (b) "Statements of General Policy or Interpretation" issued by the FDA during 1953–1956; (c) all new regulations promulgated by the Secretary of HEW during 1953–1956 dealing with definitions and standards of identity for foods; (d) cumulative tables of references to the pertinent material for the period 1938–1956 in connection with any problem under any section of the Act. The new volume contains a cumulative index to the material in the four books in this series. Those who have had the opportunity to use the earlier volumes will welcome this new addition to their reference libraries.

Topics in Microbial Chemistry. By F. M. Strong. John Wiley & Sons, Inc., New York, 1958. xi + 166 pp. 12.5 × 18.5 cm. Price $5.

This small book is based on a series of lectures by Dr. Strong and discusses antimycin (a tool of biochemical enzymologists), kinetin, and coenzyme A. Their basic chemistry, preparation, and properties are covered. Investigators will be interested in the treatment of kinetin and the kinins, a newly discovered group of plant hormones obtainable from microorganisms and physiologically active at very great dilutions. References and an index are included.

Mechanisms of Inorganic Reactions. By Fred Basolo and Ralph G. Pearson. John Wiley and Sons, Inc., New York, 1958. xi + 426 pp. 15 × 22.5 cm. Price $11.75.

This book is subtitled "A Study of Metal Complexes in Solution," and includes the modern theories in the mechanisms of substitution reactions of metal complexes, oxidation-reduction reactions, and metal ion catalysis. The crystal field theory of metal-ligand bonding is covered, and kinetic and mechanistic features of inorganic reactions are emphasized. Prior studies in coordination chemistry, stereochemistry, and reaction kinetics are useful in following this text. Chapter references are given, and indexes of authors and subjects are appended.

Standard of Japanese Pharmaceuticals. Abstracted from J. P. and J. N. F. Edited by Japan Pharmaceutical, Medical, and Dental Supply Exporters' Association. Published by Japan External Trade Recovery Organization, Tokyo, Japan, 1958. 543 pp. 15 × 21 cm.

This book presents in condensed form the monographs of drugs standardized in the Japanese Pharmacopoeia and the Japanese National Formulary. It explains briefly how drug standards are maintained and states: "These standards of Japan were established after a thorough study of U. S. P., B. P., and other official standards of foreign countries."

Soviet Pharmaceutical Research. Vol. I. Pharmaceutical Chemistry. Vol. II. Pharmacognosy. Vol. III. Medicinal Chemistry. Collected and translated into English. Consultants Bureau, Inc., 227 W. 17th St., New York 11, N. Y., 1958. 22 × 27 cm. Vol. I, 447 pp., price $95; Vol. II, 401 pp., price $90; Vol. III, 556 pp., price $100; Unit of 3 vols., price $200.

The articles that have been selected from the Russian literature and translated into English for these volumes are important for the information they contain as well as because they are unavailable in complete English form elsewhere. Volume I includes 74 papers under the general headings: Solubility; Stability; Ion-Exchange; Emulsion, Suspensions, Gels; and Miscellaneous. Volume II includes 82 articles under the general headings: Alkaloids (61 articles); Oils; Glycosides; and Miscellaneous. Volume III includes 87 articles under the general headings: Structure-Activity Relationships (10 articles), and General (77 articles). These compilations should be available to graduate students and research workers in the fields represented.

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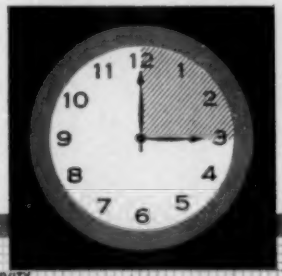

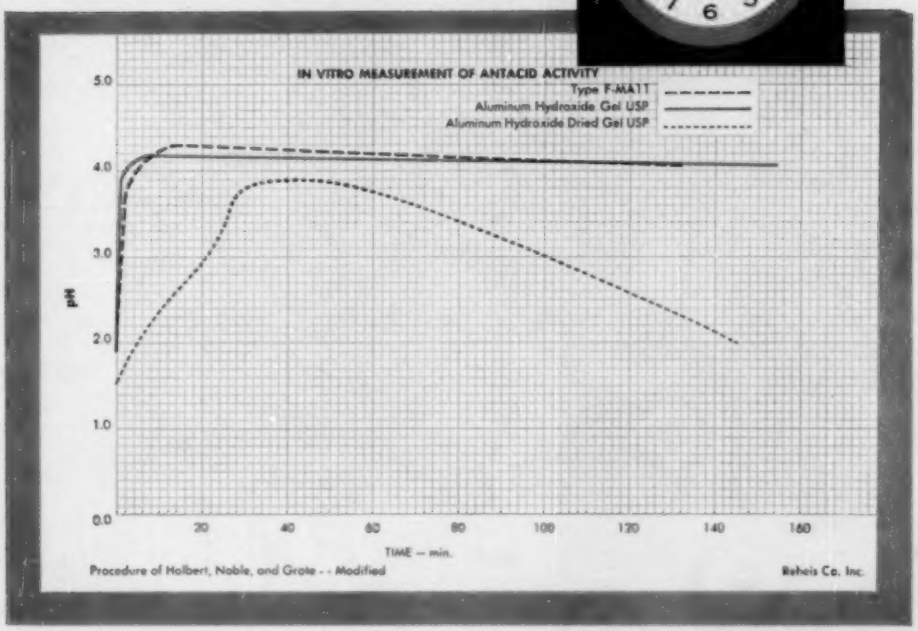

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